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(54) Title: RECOMBINANT FRAGMENTS OF THE HUMAN ACETYLCHOLINE RECEPTOR AND THEIR USE FOR TREATMENT OF MYASTHENIA GRAVIS (57) Abstract <p>Polypeptides capable of modulating the autoimmune response of an individual to human acetylcholine receptor (hAChR), more particularly polypeptides corresponding entirely or partially to the extracellular domain of hAChR α-subunit, are useful in the diagnosis and treatment of myasthenia gravis. Preferred polypeptides are polypeptides corresponding to amino acid residues 1-121 or 122-210 of the hAChR α-subunit sequence, and polypeptides corresponding to amino acid residues 1-121, 1-210 or 1-205 of the hAChR α-subunit sequence in which is inserted, between amino acid residues 58 and 59, a sequence of 25 amino acid residues encoded by the p3A exon of the hAChR α-subunit gene, and fragments, analogs, fused, soluble and denatured forms thereof. DNA molecules encoding said polypeptides are also provided.</p>		

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RECOMBINANT FRAGMENTS OF THE HUMAN ACETYLCHOLINE RECEPTOR
AND THEIR USE FOR TREATMENT OF MYASTHENIA GRAVIS

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FIELD OF THE INVENTION

The present invention relates to polypeptides capable of modulating the autoimmune response to acetylcholine receptor, and more particularly to polypeptides corresponding entirely or partially to the extracellular domain of human acetylcholine receptor α -subunit, which polypeptides are useful in the diagnosis and treatment of myasthenia gravis, and to DNA molecules encoding said polypeptides.

ABBREVIATIONS: AChR - acetylcholine receptor; α -BTX - α -bungarotoxin; EAMG - experimental autoimmune myasthenia gravis; GST - glutathione S-transferase; hAChR - human acetylcholine receptor; MG - myasthenia gravis; MIR - main immunogenic region.

BACKGROUND OF THE INVENTION

Myasthenia gravis (MG) is a human autoimmune disorder characterized by muscle weakness and fatigability. In this disease, antibodies against the acetylcholine receptor (AChR) bind to the receptor and interfere with the transmission of signals from nerve to muscle at the neuromuscular junction [Patrick and Lindstrom, 1973].

The acetylcholine receptor molecule is a transmembrane glycoprotein consisting of five homologous subunits, organized in a barrel-staves-like structure around a central cation channel, in the stoichiometry of either $\alpha_2\beta\gamma\delta$ in fetal, or $\alpha_2\beta\epsilon\delta$ in mature, muscle [Karlin, 1980; Changeux et al., 1984]. Noda et al. (1983) described the cloning and sequence analysis of human genomic DNA encoding the α -subunit precursor of muscle acetylcholine receptor, and Schoepfer et al. (1988) reported the cloning of the α -subunit cDNA from the human cell line TE671. Human muscle AChR α -subunit exists in two forms, one of which has 25 additional amino acid residues, inserted between positions 58 and 59, that are coded by the 75bp exon p3A [Beeson et al., 1990]. The α -subunit of AChR contains both the site for acetylcholine binding and the main targets for anti-AChR antibodies.

The autoimmune response in myasthenia gravis is directed mainly towards the extracellular domain of the AChR α -subunit (amino acids 1-210), and within it, primarily

towards the main immunogenic region (MIR) encompassing amino acids 61-76 [Tzartos and Lindstrom, 1980; Tzartos et al., 1987; Loutrari et al., 1992].

The involvement of antibodies directed to the MIR and to the ligand binding site of AChR in the autoimmune process can be assessed by the ability of monoclonal antibodies (mAbs) with these specificities to passively transfer experimental autoimmune myasthenia gravis (EAMG) into animals. Examples of such antibodies are mAb 198, mAb 195, mAb 202 and mAb 35 directed towards the MIR of the extracellular portion of hAChR α -subunit [Sophianos and Tzartos, 1989], and mAb 5.5 directed towards the binding site of AChR [Mochly-Rosen and Fuchs, 1981]. The anti-MIR antibodies exert their effect by crosslinking AChRs on the muscle surface thereby accelerating their degradation, and the anti-binding site mAbs by blocking and competing with acetylcholine [Souroujon et al., 1986; Asher et al., 1993; Loutrari et al., 1992a]. Anti-MIR mAbs have also been shown to accelerate the degradation of AChR in the human cell line TE671 [Loutrari et al., 1992].

MG is currently treated by acetylcholinesterase inhibitors and by non-specific immunosuppressive drugs that have deleterious side effects. It would be preferable to treat MG with a method that involves antigen-specific immunotherapy but leaves the overall immune response intact. One such strategy of specific therapy could involve the administration of derivatives of AChR that do not induce myasthenia but are capable of affecting the immunopathogenic antibodies. However, since the anti-AChR antibody repertoire in myasthenia gravis has been shown to be polyclonal and heterogeneous [Drachman, 1994], the regulation of the disease requires modulation of many antibody specificities.

Previous studies at the laboratory of the present inventors were directed towards modulating the anti-AChR response and EAMG by either denatured derivatives of Torpedo AChR, e.g. the reduced and carboxymethylated derivative, RCM-AChR [Bartfeld and Fuchs, 1978], synthetic peptides corresponding to specific regions of AChR [Souroujon et al., 1992; Souroujon et al., 1993], or mimotopes selected from an epitope library [Balass et al., 1993]. The Torpedo RCM-AChR did not induce EAMG in rabbits and was effective in suppressing the disease. However, RCM-AChR did induce EAMG in rats. The experiments carried out with the synthetic peptides and mimotopes were only partially successful in neutralizing MG autoimmune response, probably due to the incorrect folding of the short peptides that were recognized by only a portion of the anti-AChR antibodies.

MG is currently diagnosed by testing for antibodies against AChR by radioimmunoassay wherein the antigen is crude AChR extracted from human muscle or TE671 cells. This test presents some drawbacks, namely the antigen is not readily available and, in addition, the antibody titers detected are not well correlated with disease severity.

5 Thus, both a safe and effective treatment for MG, as well as a reliable and convenient diagnosis test, are much in desire.

SUMMARY OF THE INVENTION

10 It has now been found according to the present invention that polypeptides comprising sequences corresponding to the entire extracellular domain of the human AChR α -subunit, or to fragments thereof, are capable of modulating the autoimmune response to AChR. Said polypeptides, herein referred to as "biologically active" polypeptides, were found to affect the antigenic modulation of AChR in TE671 cells in vitro, and to modulate the course of EAMG in vivo; they were effective in suppressing the disease both in EAMG that was passively
15 transferred by monoclonal anti-AChR antibodies, and in EAMG that was actively induced by immunization with AChR, while they did not induce any symptoms of MG in the rat model system; they were further successful in both preventing EAMG and in suppressing an ongoing disease when administered nasally or orally to model rats .

20 Thus, the present invention provides, in one aspect, a polypeptide capable of modulating the autoimmune response of an individual to acetylcholine receptor, said polypeptide being selected from the group consisting of:

(i) a polypeptide corresponding to amino acid residues 1-210 of the human acetylcholine receptor (hAChR) α -subunit sequence depicted in Fig.1 (herein "H α 1-210"), in which is inserted, between amino acid residues 58 and 59, a sequence of 25 amino acid
25 residues encoded by the p3A exon of the hAChR α -subunit gene, depicted in Fig. 2 (herein "H α 1-210+p3A");

(ii) a polypeptide corresponding to amino acid residues 1-205 of the hAChR α -subunit sequence depicted in Fig.1 (herein "H α 1-205"), in which is inserted, between amino acid residues 58 and 59, a sequence of 25 amino acid residues encoded by the p3A
30 exon of the hAChR α -subunit gene, depicted in Fig. 2 (herein "H α 1-205+p3A");

(iii) a polypeptide corresponding to amino acid residues 1-121 of the hAChR α -subunit sequence depicted in Fig.1 (herein "H α 1-121");

(iv) a polypeptide corresponding to amino acid residues 1-121 of the hAChR α -subunit sequence depicted in Fig.1, in which is inserted, between amino acid residues 58 and 59, a sequence of 25 amino acid residues encoded by the p3A exon of the hAChR α -subunit gene, depicted in Fig.2 (herein "H α 1-121+p3A");

5 (v) a polypeptide corresponding to amino acid residues 122-210 of the hAChR α -subunit sequence depicted in Fig.1 (herein "H α 122-210");

(vi) a polypeptide as in (i) to (v) or the polypeptide H α 1-210 in which one or more amino acid residues have been added, deleted or substituted by other amino acid residues in a manner that the resulting polypeptide is capable of modulating the autoimmune response to acetylcholine receptor;

10 (vii) a fragment of a polypeptide as in (i) to (vi), which fragment is capable of modulating the autoimmune response to acetylcholine receptor;

(viii) a polypeptide comprising two or more fragments as in (vii) fused together with or without a spacer;

15 (ix) a polypeptide or a fragment as defined in (i)-(viii) or the polypeptide H α 1-210 fused to an additional polypeptide at its N- and/or C-terminal; and

(x) soluble forms, denatured forms, chemical derivatives and salts of a polypeptide or a fragment as defined in (i)-(ix).

Preferred polypeptides according to the invention are H α 1-121, H α 122-210 and, in particular, H α 1-210+p3A, H α 1-121+p3A, H α 1-205+p3A optionally fused to an additional polypeptide e.g. glutathione S-transferase (GST), and H α 1-210 similarly fused.

Preferably a fragment of H α 1-121 comprises at least the amino acid residues 61-76 of the hAChR α -subunit sequence depicted in Fig.1, and a fragment of H α 122-210 comprises at least the amino acid residues 184-210 of the hAChR α -subunit sequence depicted in Fig.1.

25 In another aspect, the invention encompasses a DNA molecule coding for a biologically active polypeptide according to the invention. Said DNA molecules may be selected from genomic DNA, cDNA or recombinant DNA or may be synthetically produced.

In particular the invention provides a DNA molecule comprising a nucleotide sequence coding for a polypeptide of the invention, said DNA molecule being selected from the group consisting of:

(i) a DNA molecule comprising the sequence of nucleotides 1 to 630, depicted in Fig.1, in which the sequence of the p3A exon of the hAChR α -subunit gene, depicted in Fig.2, is inserted between nucleotides 174 and 175;

(ii) a DNA molecule comprising the sequence of nucleotides 1 to 615, depicted in Fig.1, in which the sequence of the p3A exon of the hAChR α -subunit gene, depicted in Fig.2, is inserted between nucleotides 174 and 175;

(iii) a DNA molecule comprising the sequence of nucleotides 1 to 363 depicted in Fig.1;

(iv) a DNA molecule comprising the sequence of nucleotides 1 to 363 depicted in Fig.1, in which the sequence of the p3A exon of the hAChR α -subunit gene, depicted in Fig.2, is inserted between nucleotides 174 and 175;

(v) a DNA molecule comprising the sequence of nucleotides 364 to 630 depicted in Fig.1;

(vi) DNA molecules which are degenerate, as a result of the genetic code, to the DNA sequences of (i) to (v) and which code for a polypeptide coded for by any one of the DNA sequences of (i) to (v);

(vii) a DNA molecule having a coding nucleotide sequence which is at least 70% homologous to any one of the DNA sequences of (i) to (vi) or to the DNA sequence coding for H α 1-210;

(viii) a DNA molecule as in (i) to (v) or the DNA molecule coding for H α 1-210 in which one or more codons has been added, replaced or deleted in a manner that the polypeptide coded for by said sequence is capable of modulating the autoimmune response to acetylcholine receptor;

(ix) a fragment of a DNA molecule as in (i)-(viii) which codes for a polypeptide capable of modulating the autoimmune response to acetylcholine receptor;

(x) a DNA molecule comprising two or more fragments of (ix) fused together with or without a spacer, and which codes for a polypeptide capable of modulating the autoimmune response to acetylcholine receptor; and

(xi) a DNA molecule comprising a nucleic acid sequence as defined in (i)-(x) or the DNA sequence coding for H α 1-210 fused to additional coding DNA sequences at its 3' and/or 5' end.

Preferred DNA molecules according to the invention are those comprising the sequences of nucleotides 1-363 and 364-630, depicted in Fig.1, coding for H α 1-121 and

H α 122-210, respectively, and particularly the sequences of nucleotides 1-630, 1-615 and 1-363, depicted in Fig.1, in which the sequence of the p3A exon of the hAChR α -subunit gene, depicted in Fig.2, is inserted between nucleotides 174 and 175, said DNA molecules coding, respectively, for H α 1-210+p3A, H α 1-205+p3A and H α 1-121+p3A that comprise the additional 25 amino acid residues coded for by the p3A exon of the hAChR α -subunit gene, as well as a DNA molecule coding for H α 1-210 fused to additional coding DNA sequences e.g. the sequence coding for GST.

Preferably, a fragment DNA molecule according to the invention codes for a polypeptide comprising at least the amino acid residues 61-76 and/or 184-210 of the hAChR α -subunit sequence depicted in Fig.1.

In still other aspects, the invention provides replicable expression vehicles comprising a DNA molecule of the invention and prokaryotic or eukaryotic host cells transformed therewith.

A further aspect of the invention relates to a process for preparation of the polypeptides of the invention comprising culturing, under conditions promoting expression, host cells transformed by replicable expression vehicles comprising the DNA molecules of the invention, and isolating the expressed polypeptides.

In yet another aspect, the present invention provides pharmaceutical compositions comprising a pharmaceutically acceptable carrier and, as active ingredient, a polypeptide selected from the group consisting of the polypeptides of the invention and a polypeptide comprising the amino acid residues 1-210 of the hAChR α -subunit depicted in Fig. 1 (H α 1-210), soluble forms, denatured forms, salts and chemical derivatives thereof. The polypeptide H α 1-210 was previously described in the literature as a polypeptide which induces myasthenia gravis [Lennon et al., 1991], but the use of this polypeptide for alleviation and/or treatment of myasthenia gravis is herein disclosed for the first time.

In still another aspect, the present invention provides methods for diagnosis and for alleviation and/or treatment of myasthenia gravis using the polypeptides and pharmaceutical compositions of the invention.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 depicts the nucleotide sequence (upper line) and the amino acid sequence coded thereby (lower line) corresponding to the extracellular domain of the hAChR α -subunit (amino acid residues 1-210).

Fig. 2 depicts the nucleotide sequence (upper line) and amino acid sequence coded thereby (lower line) corresponding to the p3A exon of the hAChR α -subunit gene.

Figs. 3A-C depict Coomassie staining (3A) and Western blots with mAb 198 (3B) or mAb 5.5 (3C) of H α 1-210+p3A, H α 1-210, H α 1-121+p3A, H α 1-121 and H α 122-210 fused to glutathione S-transferase (GST) at the N-terminal (lanes 1 to 5, respectively). GST alone (lane 6) served as a control.

Fig. 4 depicts results of an ELISA assay showing binding of mAb 198 to H α 1-210+p3A (filled squares), H α 1-210 (open squares), H α 1-121+p3A (filled circles) and H α 1-121 (open circles).

Fig. 5 depicts results of an ELISA assay showing binding to H α 1-210+p3A of mAb 198 (filled squares), mAb 5.5 (open triangles), mAb 195 (filled "upside down" triangles), mAb 202 (filled "upright" triangles) and mAb 35 (open circles).

Fig. 6 depicts results of an ELISA assay demonstrating inhibition of mAb 198 (0.1 μ g/well) binding to AChR by the following polypeptides: H α 1-210+p3A (filled squares), H α 1-210 (open squares), H α 1-121+p3A (filled circles), H α 1-121 (open circles) and GST (filled triangles), at concentrations of 0.05-10 μ g/well.

Fig. 7 depicts the inhibition effect of the polypeptides of the invention on AChR degradation induced by mAb 198. TE671 cells were incubated with (a) medium, (b) 1 μ g/ml mAb 198, (c-g) 1 μ g/ml of mAb 198 preincubated with either H α 1-121 (hatched columns) or with H α 122-210 (dark columns) at concentrations of 10 (c), 25 (d), 50 (e), 100 (f) and 200 (g) μ g/ml. Residual AChR was monitored by measuring α -bungarotoxin (α -BTX) binding sites.

Fig. 8 depicts the effect of H α 1-121+p3A on AChR degradation induced by different mAbs. Residual AChR was monitored by measuring α -BTX binding sites. TE671 cells were incubated with medium alone (leftmost column) or with added mAb 198 (1 μ g/ml), mAb 35 (1 μ g/ml), mAb 195 (5 μ g/ml) or mAb 202 (5 μ g/ml) either without (dotted columns) or following preincubation of the mAbs with H α 1-121+p3A (hatched columns).

Figs. 9A-B depict the effect of nasal administration of H α 1-210+p3A and H α 1-121+p3A on T cell responses to Torpedo AChR (0.25 μ g/ml) (9A), and IL-2 production in culture (9B). Both assays were performed on cells pooled from lymph nodes taken 5 weeks after immunization with AChR from treated and control animals.

Figs. 10A-B depict the effect of nasal pretreatment on the antibody titers to H α 1-210+p3A (10A) and to rat AChR (10B), in sera from animals treated with

H α 1-210+p3A or control vehicle (GST), at 4 and 8 weeks after immunization with Torpedo AChR

Figs. 11A-B depict the effect of oral pretreatment with H α 1-210+p3A and H α 1-205+p3A on the mean clinical score of EAMG (11A) and on body weight (11B).

5 Figs. 12A-B depict the effect of oral pretreatment with H α 1-210+p3A and H α 1-205+p3A on T cell responses to Torpedo AChR (0.25 μ g/ml) (12A), and on the antibody titers to rat AChR (12B).

10 Figs. 13A-B depict the effect of oral treatment with denatured H α 1-205+p3A on an ongoing EAMG. The mean clinical score (13A) and the mean body weight change (13B) were monitored for 7 weeks following the beginning of treatment.

DETAILED DESCRIPTION OF THE INVENTION

15 Patients with the neuromuscular disease myasthenia gravis are characterized by the pathogenic autoantibodies they develop directed towards AChR [Aharonov et al., 1975]. The α -subunit of AChR appears to be the prime target for these pathogenic autoantibodies, and within it especially the extracellular domain.

20 Human muscle AChR α -subunit exists as two isoforms consisting of 437 and 462 amino acid residues [Beeson et al., 1990]. The two isoforms are identical in their amino acid composition except for a sequence of 25 additional amino acid residues inserted after position 58 in the extracellular domain of the longer variant. These additional amino acids are encoded by the 75bp exon p3A.

25 It was found according to the present invention that the polypeptides herein designated H α 1-210, H α 1-210+p3A, H α 1-121, H α 1-121+p3A, H α 1-205+p3A and H α 122-210 are capable of modulating the autoimmune response to AChR and of suppressing experimental myasthenia gravis in animal models.

The present invention thus relates to the novel polypeptides H α 1-121, H α 1-121+p3A, H α 122-210, H α 1-205+p3A and H α 1-210+p3A as well as to analogs, fragments, fused derivatives, chemical derivatives and salts thereof, and to novel analogs, fragments, fused derivatives, chemical derivatives and salts of the peptide H α 1-210.

30 Analogs according to the invention are polypeptides in which one or more amino acid residues have been added to, replaced in or deleted from the original polypeptide in a manner that the resulting polypeptide retains its biological activity. These analogs may be prepared by

known synthesis procedures and/or by genetic engineering methods, for example by expressing a DNA molecule modified by site-directed mutagenesis.

Biologically active fragments of the polypeptides encompassed by the present invention include preferably polypeptides comprising at least the amino acid residues 61 to 76 and/or 184 to 210 of the hAChR α -subunit sequence, representing, respectively, the main immunogenic region (MIR) and the acetylcholine binding site of the hAChR α -subunit. A fragment comprising at least amino acid residues 61 to 76 is a preferred fragment according to the invention. Also included in the invention are polypeptides comprising two or more fragments as mentioned above which are fused together with or without a spacer.

Chemical derivatives of polypeptides of the invention include modifications of functional groups at side chains of the amino acid residues, or at the N- and/or C-terminal groups. Examples of such derivatives include, but are not limited to, esters of carboxyl and hydroxy groups, amides of carboxyl groups generated by reaction with ammonia or with primary or secondary amines and N-acyl derivatives of free amino groups. Cyclic forms of the polypeptides containing a disulfide bridge between two cysteines residues to stabilize the molecule are also encompassed by the invention.

The salts of the polypeptides of the invention are pharmaceutically acceptable, i.e. they do not destroy the biological activity of the polypeptide, do not confer toxic properties on compositions containing them and do not induce adverse effects. The term "salts" refers to salts of carboxyl groups as well as to acid addition salts of amino groups of the polypeptide molecule.

A polypeptide of the invention, or a fragment thereof, may be fused to an additional polypeptide at its N- and/or C-terminal. For example, recombinant polypeptides were prepared where H α 1-210, H α 1-210+p3A, H α 1-121, H α 1-121+p3A or H α 122-210 were fused to glutathione S-transferase (GST) at the N-terminal, and these molecules were capable of modulating the immune response to AChR. Other polypeptides may be fused to the N- and/or C-terminal of a polypeptide of the invention provided that the fusion does not significantly impair the three dimensional structure of the resulting polypeptide in a way that prevents it from being recognized by anti-AChR antibodies.

A polypeptide according to the invention corresponding entirely or partially to the extracellular domain of the hAChR α -subunit should be capable of affecting the immunopathogenic response without inducing myasthenia gravis by itself. In order to neutralize the anti-AChR antibodies, the polypeptide may be at least partially correctly folded,

so that it will be recognized by said antibodies. Furthermore, since the anti-AChR antibody repertoire in myasthenia gravis has been shown to be polyclonal and heterogeneous [Drachman, 1994], the regulation of myasthenia gravis requires modulation of many antibody specificities. The recombinant polypeptides according to the invention have, indeed, shown to
5 have a broad specificity as demonstrated by their ability to protect AChR in TE671 cells against antigenic modulation induced by a series of anti-AChR mAbs (Fig 8) or by polyclonal anti-AChR antibodies from myasthenic rats (data not shown).

The three-dimensional arrangement of a polypeptide of the invention seems to be sometimes of crucial importance for its biological activity. It was shown in several
10 experiments (see Figs. 3B, 3C, 4 and 6) that the polypeptides comprising the additional 25 amino acid residues coded for by the exon p3A, namely H α 1-121+p3A and H α 1-210+p3A, bind to anti-AChR antibodies better than the shorter variants H α 1-121 and H α 1-210, and are also more potent in their protection effect in TE671 cells in vitro and in EAMG in vivo. Thus H α 1-121+p3A and H α 1-210+p3A are the most preferred polypeptides according to the
15 invention.

The major binding sites of the anti-AChR mAbs inhibited by the polypeptides of the invention do not seem to reside within the stretch of amino acids encoded by the p3A exon. Therefore, it seems likely that the different specificity of these mAbs towards the polypeptides with and without the p3A exon encoded sequence reflects conformational changes due to the
20 presence of said extra encoded sequence. Conformational differences could also explain why mAb 198 binds well to the polypeptides containing the p3A exon encoded sequence, but was unable to immunoprecipitate an oocyte-expressed α -subunit containing this sequence [Newland et al., 1995].

A polypeptide of the invention may be produced by means of recombinant technology
25 or synthetically employing methods well-known in the art.

Recombinant polypeptides according to the invention are prepared by culturing host cells transformed by a suitable expression vector containing a DNA molecule of the invention under conditions promoting expression, and isolating the expressed polypeptide, using standard techniques well known in the art (see, for example, Sambrook et al., 1989; Ausubel et
30 al., 1993).

Soluble forms of the polypeptides that constitute a preferred embodiment of the invention may be generated by suitable chemical modification of natural amino acid residues in the polypeptide, or by substitution of said natural amino acid residues by suitable

hydrophilic natural or non-natural amino acids. Alternatively, solubility may be induced by fusion of a polypeptide of the invention to a highly soluble polypeptide partner, such as GST, immunoglobulin or a fragment thereof, maltose binding protein (MBP), thioredoxin or influenza non-structural protein 1 (NS1). The additional polypeptide may be fused at the N- or C-terminal of the polypeptide of the invention, and it should not significantly impair the three dimensional structure of the polypeptide corresponding to the hAChR α -subunit domain.

The fused polypeptide of the invention may be used as such, or it may be subjected to further processing in which an active polypeptide of the invention is released. Insertion of a target sequence that is cleavable by specific proteases, such as V8 protease, enterokinase, thrombin or factor Xa, enables the release of the original polypeptide from the recombinant expressed fused polypeptide.

A DNA molecule according to the invention comprises a nucleotide sequence coding for a biologically active polypeptide of the invention. The DNA molecule may be from any origin including non-human sources, and may be selected from genomic DNA, cDNA, recombinant DNA, PCR-produced or synthetically produced DNA.

Preferred DNA molecules are those comprising the sequence of nucleotides 1-363 and 364-630 of the hAChR α -subunit (depicted in Fig.1) coding for H α 1-121 and H α 122-210, respectively, and particularly the sequences of nucleotides 1-630, 1-615 and 1-363 of the hAChR α -subunit in which the sequence of the p3A exon of the hAChR α -subunit gene (depicted in Fig.2) is inserted between nucleotides 174 and 175, hence coding, respectively, for H α 1-210+p3A, H α 1-205+p3A and H α 1-121+p3A.

A fused DNA molecule according to the invention comprises a nucleic acid sequence coding for a polypeptide of the invention in fusion to additional coding DNA sequences at its 3' and/or 5' end. The added DNA sequence may code for a polypeptide endowing the expressed fused polypeptide with favourable characteristics for its purification or for performing its biological activity, i.e. conferring on the original polypeptide molecule a preferred configuration or high solubility.

A DNA molecule of the invention may be directly isolated from human genomic DNA or cDNA by standard means known in the art involving subcloning genomic or cDNA fractions into a replicable vector, amplifying the subcloned fragments, detecting the relevant clones by their hybridization to the DNA molecules of the invention or fragments thereof, followed by their isolation, for example as described in Sambrook et al., eds. "Molecular

Cloning: A Laboratory Manual", 2nd ed., Cold Spring Harbor Press, 1989; and in "Current Protocols in Molecular Biology" Current Protocols, Ausubel et al., eds., 1993.

DNA molecules which are at least 70% homologous to H α 1-210, H α 1-210+p3A, H α 1-205+p3A, H α 1-121, H α 1-121+p3A or H α 122-210 may be isolated by subjecting a population of cloned genomic DNA or cDNA molecules to hybridization with the above synthesized DNA molecules or fragments thereof under stringent conditions, and isolating the hybridized clones. The term "stringent conditions" refers to hybridization and subsequent washing conditions conventionally referred to in the art as "stringent" (see Sambrook et al. and Ausubel et al., *supra*).

Alternatively, a DNA molecule of the invention may be PCR-produced as described for example in Example 1 hereinafter. In general, the PCR-production procedure comprises total RNA purification from relevant cells and generation of first strand cDNA by reverse transcriptase, using either an antisense oligonucleotide mixture or oligo (dT) as a primer. A cDNA fragment may be then amplified in a polymerase chain reaction (PCR) using appropriate sense and antisense primers flanking the target cDNA fragment. The PCR primers may include restriction sites to be used for restriction enzyme digestion followed by cloning into a suitable vector.

Cloning of a DNA molecule of the invention within an appropriate expression vehicle and expression in a suitable host cell enables production and isolation of a biologically active polypeptide or fragment thereof. For this purpose, the DNA molecule is incorporated into a plasmid or viral vector preferably capable of autonomous replication in a recipient host cell of choice. Optionally, the DNA molecule may be cloned into an expression vector in frame with additional coding sequences at its 5' and/or 3' end, e.g. the pGEX plasmid vectors that contain GST coding sequences fused upstream to the cloning site. The recombinant expression vector is then used to transform an appropriate prokaryotic or eukaryotic host cell that, under inducing conditions, expresses the polypeptide itself or fused to an additional sequence. In the latter case, insertion of a recognition site for a protease, enables at will the release of the cloned polypeptide from the additional fused polypeptide.

Vectors used in prokaryotic cells include, but are not limited to, plasmids capable of replication in *E. coli*, for example, pGEX, and bacteriophage vectors such as λ gt11, λ gt18-23, M13 derived vectors etc.

A vector construct containing the DNA molecule of the invention is then introduced into an appropriate host cell by any of a variety of suitable means known in the art, such as

transformation, transfection, lipofection, conjugation, protoplast fusion, electroporation, calcium phosphate precipitation, direct microinjection, etc.

Suitable host cells useful in the invention are prokaryotic cells which include, but are not limited to *E. Coli*, and more preferably, eukaryotic hosts which include, but are not limited to, yeast cells such as *Saccharomyces cerevisiae*, or insect cell lines, for example *Spodoptera frugiperda* (Sf9) cells which are commonly used with the baculovirus expression system, or mammalian cells such as Chinese hamster ovary (CHO) cell lines.

Eukaryotic cells are the preferred hosts in expression systems for producing the polypeptides of the invention since they can perform the correct post-translational processing to confer the right conformation on said polypeptides. However, since partially correctly folded polypeptides may also be biologically active, prokaryotic expression systems may also be useful, especially for the production of large amount of polypeptides.

In another aspect, the present invention relates to pharmaceutical compositions comprising a pharmaceutically acceptable carrier and, as active ingredient, a polypeptide selected from a polypeptides of the invention and a polypeptide comprising the amino acid residues 1-210 of the hAChR α -subunit depicted in Fig.1, soluble and denatured forms, salts and chemical derivatives thereof.

The pharmaceutical compositions are for use in the alleviation and/or treatment of myasthenia gravis and may be in any suitable form for administration of polypeptides known in the art, e.g. by injection, inhalation, orally, nasally, etc.

Appropriate pharmaceutically acceptable carriers include physiological carriers, such as water and oils and excipients such as stabilizers and preservative agents. Saline solutions and aqueous dextrose and glycerol solution are suitable for injectable solutions. The active ingredient may also be prepared as a lyophilized dry compound, possibly as a salt, or as a conjugate with a solid carrier/support such as dextran, natural and modified celluloses, etc. The pharmaceutically acceptable carrier of choice will be determined depending on the route the pharmaceutical composition will be administered.

The dosage of the polypeptide and the schedule of the treatment should depend on the route of administration, the patient condition, age and genetic background and will be determined by a skilled professional person. For example, based on animal studies, it was found that dosage ranges of about 1.4 μg - 14 mg and 0.14 μg - 0.7 mg/ Kg human body weight are suitable for oral and nasal administration, respectively, in humans.

The invention further provides a method for alleviation or treatment of myasthenia gravis which comprises administering to an individual in need thereof an effective amount of a polypeptide selected from a polypeptide of the invention and the polypeptide H α 1-210, a soluble form, a denatured form, a chemical derivative or a salt thereof.

5 In contrast to the current methods of treatment of MG using non-specific immunosuppressive drugs, such as steroids, azathioprine or cyclosporine, the method of present invention is directed to an antigen-specific immunotherapy strategy thus suppressing only the adverse autoimmune responses while leaving the overall immune system of the patient intact.

10 Preferred routes of administration of the polypeptides according to the invention are the nasal and oral routes.

Nasal tolerization has several advantages as a treatment modality in comparison with oral tolerization: it requires smaller doses of antigen, is more convenient to use and does not require soybean trypsin inhibitor (STI) used in oral tolerance to inhibit the degradation of the antigen in the gastrointestinal tract. Some successful attempts to modulate experimental autoimmune diseases in animal models by nasal administration of the autoantigen have been recently reported. Thus, Weiner et al. [1994] showed that inhalation of aerosols containing myelin basic protein (MBP) abrogated the clinical symptoms of EAE and significantly reduced the CNS inflammation, DTH reaction and antibody titer to MBP; Dick et al. [1993] reported that nasal administration of retinal extract inhibited the induction of experimental allergic uveitis (EAU) by immunization with this extract; and Ma et al. [1995] demonstrated that nasal administration of the antigen Torpedo AChR diminished the incidence and severity of clinical muscle weakness characteristic of EAMG following immunization with said antigen.

25 The pharmaceutical compositions of the present invention are also useful for diagnosis of myasthenia gravis whereby anti-AChR antibodies in the serum of a patient are determined by employing one or more polypeptides of the invention as the test antigen and bound anti-AChR antibody titers indicate the presence of myasthenia gravis.

30 For the diagnostic test, a serum aliquote of a patient is brought in contact with one or more polypeptides, incubated for about 1 h to overnight at 4°-37°C, followed by the determination of the amount of anti-AChR antibodies bound to the polypeptides by quantitative detection assays known in the art.

In one embodiment, the diagnostic test is carried out with immobilized polypeptides in an assay comprising the following steps:

(i) immobilization of one or more polypeptides corresponding entirely or partially to the extracellular domain of human acetylcholine receptor on a suitable solid support;

5 (ii) incubation of said immobilized polypeptides of (i) with a serum sample from a patient for 1 h to overnight at 4°-37°C; and

(iii) determination of the amount of the anti-AChR antibodies bound to the immobilized polypeptides fragments,

whereby detection of anti-AChR titers indicates the presence of myasthenia gravis.

10 The detection of the anti-AChR antibodies may be carried out with labeled anti-human antibodies or labeled Staphilococcus protein A. The label may be a radioactive or fluorescent tag, an enzyme conjugate or another biological recognition tag. Examples of radioactive tags are radioactive isotopes such as ¹²⁵I, ³⁵S, ³²P, ³H, ¹⁴C etc, which are detected by a scintillation or a γ -counter or by autoradiography. Fluorescent tags are derived from fluorescent
15 compounds such as fluorescein, isothiocyanate, rhodamine, phycoerythrin, phycocyanin, allophycocyanin, o-phthaldehyde and fluorescamine, and are detected by exposure of the bound fluorescent labeled antibody to light of the proper wavelength and monitoring the fluorescence.

Enzyme conjugates useful for detection purposes include, but are not limited to,
20 maleate dehydrogenase, yeast alcohol dehydrogenase, horseradish peroxidase, alkaline phosphatase, beta-galactosidase, catalase and glucose-6-phosphate dehydrogenase. These enzymes are conjugated to the antibody or to protein A and can be monitored by the product they produce when exposed to the appropriate substrate. The chemical moiety thus released can be detected, for example, by chemiluminescence reaction or by spectrophotometry,
25 fluorometry or visual means.

Diagnostic methods based on recognition of biological tags include, for example, coupling of protein A or of the anti-human antibodies to biotin. The biotinylated molecules then can be detected by avidin or streptavidin coupled to a fluorescent compound, to an enzyme such as peroxidase or to a radioactive isotope and the like.

30 In another embodiment, the diagnostic test is carried out with one or more soluble polypeptides pre-labeled by one of the foregoing labels and tags, whereby anti-AChR antibodies of the patient's serum bound to the polypeptides are separated from the free antigen

by precipitation of the antigen-antibody complex by Staphilococcus protein A or anti-human antibodies, and anti-AChR titers are determined as described above.

The diagnostic assays according to the invention have the advantage of avoiding the need to extract the antigen from human tissues or cells, and also provides a more reproducible and safe way for MG detection. The use as antigens of polypeptides that recognize sub-populations of MG-related antibodies further provides a better means for correlating anti-AChR titers with disease severity.

The invention will now be illustrated by the following non-limiting examples and accompanying drawings.

EXAMPLES

MATERIALS AND METHODS

i) Monoclonal antibodies (mAb).

The following monoclonal antibodies were used: mAb directed towards the main immunogenic region (MIR) of the extracellular portion of the hAChR α -subunit [Sophianos and Tzartos, 1989]: mAb 198, mAb 195 and mAb 202 elicited in rats against human muscle AChR, and mAb 35 elicited in rats against electric eel AChR, but cross-reacted with AChR from other species, including human; and mAb 5.5 directed towards the binding site of AChR from other species, including human [Mochly-Rosen and Fuchs, 1981], elicited in mouse against Torpedo AChR.

ii) Antibody binding assays.

Binding of antibodies to AChR or to recombinant polypeptides corresponding entirely or partially to the extracellular domain of the hAChR α -subunit was analyzed by ELISA. Wells of microtiter plates (Maxisorb, Nunc, Neptune, NJ) were coated by incubation overnight at 4°C with either Torpedo AChR (1 μ g in 100 μ l of phosphate-buffered saline (PBS)), or with one of the recombinant polypeptides of the invention (2 μ g in 100 μ l of 50 mM Tris buffer pH 8.0). Coated plates were washed three times with PBS containing 0.05% Tween-20, then wells were blocked by incubation for 1 h at room temperature (R.T.) with 1% bovine serum albumine (BSA) and 1% hemoglobin in PBS, and the coated blocked plates were then washed and incubated overnight at 4°C with different amounts of antibody.

For inhibition experiments, each well was coated with 1 μ g of Torpedo AChR and a polypeptide of the invention was preincubated with the mAb of choice for 30 min at R.T.

before addition to the AChR-coated well. Following a washing step, bound mAb was determined by incubation for 1 h at R.T. with 1:5000 dilution of alkaline phosphatase (AP)-conjugated goat anti-mouse Igs (Jackson ImmunoResearch Labs, Inc., or Biomakor, Ness-Ziona, Israel). The bound antibody was detected by the enzymatic activity of AP using N-para-nitrophenyl-phosphate as a substrate and determining by a microtiter plate reader at 405 nm the color developed after about 40 min.

iii) Determination of AChR content

AChR content was determined by measuring α -bungarotoxin (α -BTX) binding sites. Tested samples were derived from (a) muscle preparations or from (b) cells grown in a tissue culture.

a) For the muscle preparation, the procedure described by Souroujon et al. [1985] was essentially followed. Briefly, muscle tissue was removed and homogenized in a Sorvall omnimixer for 2 min. at full speed. Two volumes of Tris-HCl buffer, pH 7.5, containing 0.1 M NaCl, 1 mM EDTA, 0.1 mM PMSF and 0.5 mM NaN₃, were used for homogenization. Homogenates were then centrifuged at 48,000 x g for 1 h, washed once and recentrifuged as above. The homogenates were stirred overnight at 4°C in 2 volumes of the above Tris buffer containing Triton X-100 at a final concentration of 1%. The mixture was then centrifuged for 1 h at 100,000 x g in a Beckman ultracentrifuge and the recovered supernatant was stored at -70°C. The AChR in the Triton extracts was determined by measuring the amount of ¹²⁵I- α -BTX that coprecipitated with the receptor in ammonium sulfate at 35% saturation. Unbound toxin was removed by filtration through GF/C filters, and radioactivity retained on filters, i.e. toxin bound to receptor, was measured in a γ -counter.

b) For determination of the AChR content in TE671 cells grown in tissue culture, ¹²⁵I- α -BTX (final concentration about 2×10^{-9} M; 10⁶ cpm) was added to a confluent cell culture in a 30 mm plate and incubated for 1 h at 37°C. The cells were then washed four times with PBS, released with 1N NaOH and cell-bound radioactivity was evaluated in a γ -counter, after deducting cpm in a control test tube containing an excess of unlabeled α -BTX (final concentration 10⁻⁶ M).

iv) Western blots

Electrophoresis of recombinant polypeptides corresponding to the entire or partial extracellular domain of the hAChR α -subunit and their blotting were performed essentially as described [Wilson et al., 1985; Neumann et al., 1985]. The polypeptides were electrophoresed

in 10% polyacrylamide gels and transferred to a nitrocellulose membrane. The membrane was preincubated in PBS containing 0.5% hemoglobin for 1h at R.T. before addition of 10µg/ml mAbs and incubation was carried out for additional 3 h at 37°C. The membranes were washed 4 times with PBS, once with PBS containing 0.5 % Triton X-100 and then incubated for 1h at 37°C with ¹²⁵I-goat-anti-mouse Ig. After five washes, the blots were exposed to an X-ray sensitive film.

v) Antigenic modulation in TE671 cells

Antigenic modulation experiments were performed in 30-mm 12-well plates using TE671 cell cultures. Cells (2×10^4) were plated in Dulbecco Modified Eagles medium (DMEM) containing 2 mM L-glutamine, 10% fetal calf serum (FCS) and antibiotics (100 U/ml penicillin, 100 µg/ml streptomycin and 250 ng/ml amphotericin B), and grown to confluency for 72 h. The antibodies were added in triplicate to culture wells at a concentration of 1 µg/ml (and for mAbs 195 and 202 also at 5 µg/ml) for 3 h. At the end of the incubation, ¹²⁵I-α-BTX was added at a final concentration of 2×10^{-9} M (10^5 cpm) for an additional hour. AChR content was determined by measuring ¹²⁵I-α-BTX binding, as described in section (iii) above.

In order to test the effect of the polypeptides of the invention on the antigenic modulation induced by the antibodies, the mAbs were preincubated for 1 h at 37°C with said polypeptides (at concentrations of 10-200 µg/ml, as indicated), before their addition to the cell cultures, and the assay continued as described in section (ii) above.

vi) Passive transfer of EAMG to rats.

Lewis female rats (6 weeks old, approximate weight 120 g) were used for passive transfer experiments, as previously described [Asher et al., 1993]. For the induction of EAMG, 80 µg of the anti-MIR mAb 198 in 1 ml PBS were injected i.p. into each rat. The tested polypeptide (1 mg) was preincubated with mAb 198 for 30 min at R.T., prior to the injection into rats. The rats were observed for myasthenic symptoms and body weight. At 48 h after the administration of mAb, the animals were sacrificed and their leg muscles were removed for determination of the AChR content according to section (iii) above.

vii) Induction of EAMG and clinical evaluation

Animals were injected once in the hind foot pads with 40 µg of Torpedo AChR emulsified in complete Freund adjuvant (CFA) containing 1 mg/rat *Mycobacterium Tuberculosis* (Difco Lab., Detroit, MI). Experimental animals were weighted every week.

Clinical EAMG was evaluated as follows: grade 0, no weakness or fatigability; grade 1, weak grip, fatigability; grade 2, weakness, hunched posture at rest, decrease in body weight, tremolousness; grade 3, severe weakness, marked decrease in body weight, moribund; grade 4: dead. Animals were evaluated weekly up to 7-9 weeks after immunization with Torpedo AChR. Blood samples were obtained from the retroorbital plexus.

viii) Lymphocyte proliferation assay

Popliteal lymph nodes were aseptically removed and single cell suspensions were prepared in RPMI with 10 mM HEPES. An in vitro T-lymphocyte proliferative assay in response to AChR and the different polypeptides of the invention was performed as follows:

- 10 Lymph node cells were suspended in RPMI at pH 7.4 containing 10 mM HEPES, supplemented with 2 mM L-glutamine, 1 mM sodium pyruvate, 0.1 mM nonessential amino acids, 5×10^{-5} M β -mercaptoethanol and 0.5% normal rat serum, and plated in 96-well flat bottom plates (Corning; 5×10^5 cells/well). Increasing concentrations of antigen (0.25 to 10 μ g/ml of AChR and 10 to 100 μ g/ml of a recombinant polypeptide of the invention), were then
- 15 added to each well. Plates were incubated at 37°C, in 7.5% CO₂ and 90% humidity. Proliferation was assayed after 3 days by measuring incorporation of thymidine-methyl-[³H] into cells. Essentially, the cells were incubated with thymidine-methyl-[³H] (Rotem Ind. Ltd, Beer Sheva, Israel; 0.5 mCi/2.5ml) for 24 h and then harvested and counted for radioactivity. Results are presented as incorporated cpm following subtraction of cpm in the presence of
- 20 medium alone.

EXAMPLES

Example 1: Preparation of recombinant DNA molecules

- DNA molecules encoding the biologically active polypeptides H α 1-210, H α 1-121, H α 122-210, H α 1-205+p3A, H α 1-210+p3A and H α 1-121+p3A were synthesized as follows:

- Total RNA was prepared as described [Asher, 1988] from the human TE671 cell line, which expresses the human muscle type nicotinic AChR [Schoepfer et al., 1988]. Preparation of cDNA and the polymerase chain reaction (PCR) were performed as described [Barchan et al., 1992]. The primers employed to amplify cDNA fragments corresponding to the hAChR α -subunit residue 1-210 (H α 1-210), with or without the p3A exon (H α 1-210+p3A) [Beeson et al., 1990], were constructed with sites that enabled cloning into the fusion protein expression vector pGEX-2T. The primer at the 5' end, CCGGATCCGAACATGAGACC,

corresponds to amino acid residues 1-5 of the human AChR α -subunit sequence (nucleotides coding for the first residue are bold), and had a BamHI site (underlined). The primer at the 3' end had an EcoRI site (underlined) and was complementary to the DNA sequence coding for amino acid residues 206-210, CGGAATTCCAGGCGCTGCATGAC.

5 In a similar way the shorter clones H α 1-121, H α 1-121+p3A and H α 122-210 were derived by PCR using the above-mentioned H α 1-210 and H α 1-210+p3A clones as templates. For obtaining the two DNA molecules corresponding to amino acid residues 1-121 (with and without the amino acid residues coded by the p3A exon), a primer complementary to the DNA sequence coding for amino acid residues 116-121 with an EcoRI site (underlined)
10 CGGAATTCTGAGGTGTCCACGTGAT, was used at the 3' end. For the 5' end, the primer described above corresponding to amino acid residues 1-5 was used. For cloning of the DNA coding for H α 122-210, the primer CCGGATCCGCCATCTTTAAAGC was used at the 5' end. This primer corresponds to amino acid residues 122-126 (nucleotides coding for residue 122 are in bold) and contains a BamHI site (underlined). The primer used at the 3' end was the
15 same as described above for the DNA molecule coding for H α 1-210 (complementary to residues 206-210). The PCR amplified DNA sequences were subcloned into the BamHI-EcoRI sites of pGEX-2T expression vector (Pharmacia) [Smith and Johnson, 1988], in frame with the GST-coding DNA sequences at the 5' end.

The clone H α 1-205+p3A was derived by PCR, using as template the cDNA of hAChR
20 from the TE671 cell line. The primer at the 5' end (GGCCATGGGCTCCGAACATGAGACC) corresponded to amino acid residues 1-5 was designed in a way that enabled cloning into a pET8C-derived expression vector by adding a restriction site for NCO I (underlined) the initiation codon ATG. The primer at the 3' end (CCGGATCCTCAAAAGTGRTAGGTGATRTC, where R=A or G) corresponded to the
25 complementary sequence of amino acid residues 200-205, and contained a restriction site for BamHI (underlined) and a stop codon.

All the cloned DNA molecules were sequenced in order to verify their sequence and then used to produce the recombinant polypeptides.

30 Example 2: Preparation of recombinant polypeptides

The different recombinant DNA molecules subcloned in pGEX-2T plasmid prepared in Example 1 were used to transform competent E. coli cells (strains JM101 or XL1-blue). The transformed bacteria were grown overnight in LB medium containing ampicillin, then diluted

1:150 in the medium and further grown for additional 3-5 h. Induction of fused polypeptide expression was achieved by adding 0.5 mM IPTG (isopropyl β -D-thiogalactopyranoside) for 2 h. After expression, the bacterial suspension was centrifuged, cells were lysed by freezing and thawing the pellet and resuspended in PBS (10 ml). The preparation was sonicated for five
5 15-sec periods, and centrifuged for 15 min at 27,000 x g. The expressed recombinant fused polypeptides were localized in the precipitate, probably in inclusion bodies. The fused polypeptides were solubilized in 1 ml of 9 M urea, the non-soluble fraction was removed by centrifugation for 45 min at 27,000 x g, and the supernatant was diluted in 10 ml of 50 mM Tris buffer, pH 8.0 and dialyzed against the same buffer for 48 h with several changes. After
10 ultracentrifugation for 30 min at 100,000 x g, the supernatant was divided into aliquots for storage at -80°C. The protein concentration, determined by the Lowry method, was 1-3 mg/ml, with a yield of 20-50 mg of total protein from one liter of bacterial suspension. The GST-fused polypeptides were isolated using a substrate affinity column according to Smith and Johnson, 1988. A Coomassie brilliant blue staining of the expressed GST-fused polypeptides run on
15 10% polyacrylamide gel is shown in Fig. 3A: from left to right, lanes 1-6, H α 1-210+p3A, H α 1-210, H α 1-121+p3A, H α 1-121, H α 122-210 and GST, appearing to have MW of 52.5, 50.0, 43.7, 41.2, 37.8 and 29.0 kD, respectively, in agreement with the expected MW calculated based on the encoded amino acid sequences of these polypeptides (see Fig.1 and Fig.2).

20 Expression of H α 1-205+p3A in the pET8C expression system was performed in a similar procedure using E. coli BL21 strain.

Example 3: Immunochemical characterization of the recombinant polypeptides

25 The recombinant polypeptides of Example 2 were further characterized by their binding to various anti-AChR mAbs as assayed by both Western blots (Fig. 3B- mAb 198; Fig. 3C- mAb 5.5) and by ELISA (Fig. 4 and Fig. 5).

The recombinant polypeptides (20 μ g each) were electrophoresed, blotted onto nitrocellulose membrane, and incubated with different mAbs as described in Materials and Methods, section (iv). Fig. 3B shows that mAb 198, which is directed to the MIR, bound to
30 the polypeptide corresponding to the entire extracellular portion of the hAChR α -subunit (H α 1-210) and to its shorter derivative (H α 1-121), that contains the MIR, as well as to their variants including the additional p3A encoded sequence H α 1-210+p3A and H α 1-121+p3A.

As expected, mAb 198 did not bind to H α 122-210, which does not include MIR, or to the GST protein itself.

The mAb 5.5, which is directed to the binding site of AChR [Mochly-Rosen and Fuchs, 1981], bound to H α 1-210, H α 1-210+p3A and to H α 122-210, all including the binding site, but it did not bind to H α 1-121, H α 1-121+p3A nor to the GST protein (Fig. 3C). As shown, both mAb 198 and mAb 5.5 bound better to the variants containing the sequence encoded by the p3A exon.

The binding of mAb 198 to the polypeptides of the invention was also determined in ELISA carried out as described in Materials and Methods section (ii), and the results are shown in Fig. 4. In this assay, as in the Western blot, mAb 198 bound better to the polypeptides H α 1-210+p3A and H α 1-121+p3A (filled symbols). Therefore, these longer variants were used in further studies. Three other anti-MIR mAbs (mAb 195, mAb 202 and mAb 35) bound to a lesser extent than mAb 198 to all tested polypeptides (not shown).

Fig. 5 illustrates the binding of various mAbs to H α 1-210+p3A: Mab 198 (filled squares) showed a very strong binding. Mab 35, which is directed against the MIR and is known to depend on the native conformation of AChR, showed very low binding to the tested polypeptides of the invention (open circles). Mab 5.5 which also depends on the native conformation of AChR, bound well to the tested polypeptides in Western blots (Fig. 3C), but to a much lesser extent than mAb 198 in ELISA (open triangles). This poor binding of mAbs 35 and 5.5 may indicate that when bound to ELISA plates only a small fraction of the recombinant polypeptide is properly folded.

Based on the results of the binding experiments in ELISA, the next step was to test whether the polypeptides of the invention bind to the mAbs also in solution. For that, the ability of the various recombinant polypeptides to inhibit the binding of mAb 198 to Torpedo AChR was tested in ELISA. As shown in Fig 6, H α 1-210+p3A (filled squares) and H α 1-121+p3A (filled circles) inhibited this binding, with IC₅₀ values of 1.8×10^{-7} M and 1×10^{-7} M, respectively, whereas the GST protein (filled triangles) did not, indicating that the solubilized recombinant fused polypeptides may indeed bind to mAb 198 also in solution. As shown above (Figs. 3B and 4), the variants containing the additional 25 amino acid residues encoded by the p3A exon were more potent in inhibiting mAb 198 binding to AChR than their counterparts lacking this 25-mer.

Example 4: Effect of the polypeptides on antigenic modulation of AChR in TE671 cells

Muscle AChR loss in myasthenia gravis is caused by accelerated degradation of the receptor, brought about by anti-AChR antibodies, a great portion of which are directed to the MIR. This activity of the antibodies can be demonstrated *in vitro* in cell cultures such as the human cell line TE671. This human medulloblastoma-derived cell line expresses a functional AChR which binds α -BTX and has the α -subunit of the muscle-type AChR. The ability of the recombinant polypeptides H α 1-210 and H α 1-121 to protect the AChR on TE671 cells against accelerated degradation of AChR induced by specific anti-AChR α -subunit mAbs, was examined as follows: Anti-MIR mAbs were preincubated for 1 h at 37°C with several concentrations of the recombinant polypeptide and then added to the cells. As a control, the mAbs were preincubated with GST or with the H α 122-210 polypeptide that does not include the MIR. The inhibition effect of H α 1-121 on AChR degradation induced by mAb 198 measured as residual α -BTX binding sites, is illustrated in Fig. 7. MAb 198 causes a reduction of 41% in residual AChR following 3 h incubation with the cells (Fig. 7, lane b). Preincubation with increasing concentrations of H α 1-121 had a dose dependent protection effect against the degradation induced by mAb 198 (Fig. 7, c-g, hatched columns). At a concentration of 100 μ g/ml of H α 1-121 the TE671 cells were completely protected against the accelerated AChR degradation by mAb 198. Preincubation of mAb 198 with H α 122-210, which does not contain the MIR, did not affect the antigenic modulation induced by mAb 198 and did not block AChR degradation (Fig. 7, c-g, dark columns). H α 1-210, corresponding to the entire extracellular α -subunit domain, had the same effect as the shorter fragment H α 1-121 (data not shown).

Results of a comparable experiment carried out with other anti-AChR mAbs are shown in Fig. 8. The polypeptide H α 1-121 had a similar protection effect against AChR degradation induced by two other anti-MIR mAbs, mAb 195 and mAb 202, but had a much smaller effect on mAb 35-induced AChR degradation, possibly because of the weak binding of this antibody to H α 1-121 in solution (see Fig. 5).

Example 5: Modulation by the polypeptides of EAMG passively transferred by mAb 198

The effect of the polypeptides of the invention was also examined *in vivo* in a well-established animal model disease for myasthenia gravis, designated experimental autoimmune myasthenia gravis (EAMG) [Lindstrom et al., 1976 and 1976a]. In animals such

as rabbits, mice, guinea-pigs, monkeys and rats, EAMG can be either passively transferred by anti-AChR antibodies, or actively induced by AChR. In both cases, the treated animals show chronic symptoms of the MG disease, i.e. show general weakness, have a hunched posture, develop a flaccid paralysis of the hind limbs, have difficulties in breathing, in swallowing and
5 in reaching food and water supplied to them, all of which result in weight loss. The animals die from respiratory insufficiency, malnutrition and dehydration. In rats, two distinct episodes of weakness occur, especially after immunization with Torpedo AChR in combination with *Mycobacterium tuberculosis* (killed) H37 Ra, with an acute phase starting 8-10 days after immunization and a chronic phase starting 3-5 weeks later.

Table 1: Recombinant fragments modulate experimental myasthenia passively transferred by a monoclonal anti AChR antibody.

Treatment	Anti-AChR mAb 198	Myasthenic symptoms	AChR content* fmoles/mg prot.	% of control
-	-	-	39.9±6.3	100
-	+	+	19.2±3.5	48
Hα1-121	+	-	38.8±6.9	97
Hα122-210	+	+	24.5±2.4	61
GST	+	+	19.2±4.5	48
BSA	+	+	21.4±2.4	53

*Muscle AChR content was determined by α-bungarotoxin binding to AChR present in Triton X-100 extracts from rat leg muscles, 48 h after Ig administration. The values (mean±SEM) are averages derived from at least three different animals.

EAMG was passively transferred in rats by mAb 198. The disease was induced within 24-48 h following administration of the antibody [Asher et al., 1993]. Muscle AChR content was determined by α -bungarotoxin binding to AChR present in Triton X-100 extracts from rat leg muscles, 48 h after the mAb administration. As previously reported, the myasthenic symptoms were accompanied by a marked reduction in the muscle AChR content (48% of normal control; Table 1). In order to examine the effect of the polypeptides of the invention on the disease symptoms, mAb 198 was preincubated with a 30 fold molar excess of recombinant polypeptides of the invention, or with either GST or BSA as controls, prior to its injection into rats.

As shown in Table 1, the muscle AChR content in the EAMG-induced rats was reduced to 48% of AChR content of control untreated rats. The recombinant polypeptides of the invention were able to modulate in vivo muscle AChR loss and to decrease significantly clinical symptoms of EAMG. It was shown that preincubation of mAb 198 with H α 1-121+p3A prior to its injection into rats, prevented the appearance of myasthenic symptoms. The protected rats had a normal muscle AChR content (97% of control). Similar results were obtained with the H α 1-210+p3A polypeptide (data not shown). On the other hand, preincubation with either H α 122-210+p3A or with GST or BSA did not affect the muscle AChR content significantly (61, 48 and 53% of control, respectively) and did not prevent myasthenic symptoms. Administration of H α 1-121+p3A and H α 122-210+p3A alone did not induce any myasthenic symptoms in rats.

Interestingly, similar protection effect by H α 1-121+p3A and H α 1-210+p3A was demonstrated when the recombinant polypeptide was injected together with mAb 198 without preincubation, or even two hours after the administration of mAb 198 (data not shown).

Example 6: Protective effects of nasal administration of the polypeptides of the invention on actively induced EAMG in rats

H α 1-210+p3A, H α 1-121+p3A and H α 122-210 fused with GST were expressed and solubilized as described in Example 2. Nasal tolerance was induced in rats by administration of a daily dose of 2.5 μ g of each of said fused polypeptides in 30 μ l PBS into each rat nostril, over a period of ten consecutive days. Three days later the rats were immunized with Torpedo AChR (40 μ g/rat) injected into the footpads, in Complete Freund's Adjuvant supplemented with 1 mg of Mycobacterium tuberculosis H37RA (DIFCO). Control rats received GST instead of the recombinant polypeptide. Clinical symptoms of EAMG disease, as well as body

weight, were monitored weekly. The results of the experiment are summarized in Table 2, showing that all three tested polypeptides had a protective effect in the rats.

Rats treated intranasally with either of the three recombinant fragments, before immunization with Torpedo AChR, were protected against EAMG, as assessed by clinical symptoms of EAMG as well as by weight loss and muscle AChR content as summarized in Table 2. 67%, 56% and 34% of the rats pretreated with H α 1-210+p3A, H α 1-121+p3A and H α 122-210+p3A, respectively, were completely protected and did not develop clinical symptoms of EAMG, and the other rats in these groups were partially protected and had milder symptoms. On the other hand, all rats in the control, GST-pretreated group, were sick. As shown in Table 2, there was a marked effect of the treatment on the weight of the rats. Whereas rats in the control, GST-treated group, exhibited a notable decrease in body weight (12.8 ± 9.2 g) characteristic to EAMG, between 3 weeks and 7 weeks following AChR injection, rats in groups pretreated with AChR fragments increased significantly in their body weight. The protective effect of the nasal treatment was also evident from the receptor content data. As seen in Table 2, there was a decrease of about 55% in AChR content in the control-GST treated rats, and only 11% decrease in AChR content in rats pretreated with H α 1-210+p3A. The recombinant fragments themselves had no myasthenogenic effects under the conditions employed for treatment. Protection against EAMG by nasal administration of the polypeptides of the invention was accompanied by a reduction in the proliferative T-cell response and IL-2 production in response to AChR (Fig. 9), and in the antibody titers to both H α 1-210+p3A and to self, rat AChR (Fig. 10).

Table 2: The effect of intranasal treatment with human recombinant AChR fragments on EAMG in rats.

Treatment	Clinical score ^a				Healthy rats %	Δ weight 3w to 7w gr	AChR content	
	0 4	1	2	3			fmoles/mg prot.	%
	No/total							
Control vehicle (GST)	0/10 2/10	2/10	2/10	4/10	0	-12.8 \pm 9.2	17.5 \pm 4.1	44
H α 122-210	3/9	3/9	1/9	1/9	33	+9.2 \pm 8.6	14.9 \pm 1.7	38
H α 1-121 +p3A	5/9	1/9	2/9	0/9	56	+13.6 \pm 2.5	29.6 \pm 4.5	75
H α 1-210 +p3A	6/9	1/9	1/9	0/9	67	+15.0 \pm 6.3	35.0 \pm 3.4	89
Normal rats							39.5 \pm 2.5	100

^aEvaluated 7 weeks after the induction of EAMG.

Example 7: Suppressive effects of nasal administration of the polypeptides of the invention in an ongoing EAMG.

5 In order to evaluate the potential of the polypeptides of the invention to affect an ongoing disease, nasal administration of H α 1-210+p3A was initiated 7 days after the induction of EAMG by immunization with Torpedo AChR. At this time rats are known to be at the first, acute phase of EAMG. Other than the time of initiation, the protocol for the nasal administration was as in Example 6.

10 As summarized in Table 3, suppression of EAMG was observed also when nasal treatment with H α 1-210+p3A was initiated after the induction of EAMG. Among the rats treated intranasally with H α 1-21+p3A, 30% were disease-free for at least 8 weeks following induction of EAMG, and in the other rats in the group the symptoms seemed to be milder. There was also an effect of the nasal treatment on the receptor content. As seen in Table 3, there was a loss of 68% in the AChR content in the control (treated intranasally with ovalbumin) rats, and only a 20% loss in the rats treated intranasally with H α 1-210+p3A.

Table 3: The effect of intranasal treatment with human recombinant AChR fragment H α 1-210 +p3A on ongoing EAMG in rats.

Treatment ^a	Clinical score ^b				Healthy rats %	AChR fmoles/mg protein	content %
	0	1	2	3			
	No/total						
Control vehicle (OVA)	0/10	2/10	4/10	3/10	0	11 \pm 1	32
H α 1-210 +p3A	1/10	3/10	3/10	3/10	30	27 \pm 8.5	80
Normal rats						34 \pm 8.5	100

^aNasal administration was initiated 7 days after induction of EAMG by immunization with AChR and was continued for 12 consecutive days.

^bClinical evaluation was made 10 weeks after induction of EAMG.

Example 8: Effects of oral administration of the polypeptides of the invention on EAMG in rats.

The potential of oral administration of the polypeptides of the invention to prevent EAMG was first investigated. Two recombinant preparations of the extracellular domain of human AChR α -subunit were employed for oral tolerization: H α 1-210+p3A (fused with GST), and the extracellular domain itself (H α 1-205+p3A) expressed in the pET8C expression system with no fusion protein. Rats were fed 5 times with three days interval, each time with 0.6 mg of the recombinant fragment per rat, and AChR was injected in CFA to induce EAMG, three days after the last feeding. Rats were followed clinically, as well as for weight loss for 8 weeks after EAMG induction. As shown in Fig. 11, oral feeding with either GST-fused H α 1-210+p3A or with H α 1-205+p3A had a significant protective effect on the clinical symptoms of EAMG for at least 8 weeks. The values represent the average clinical score in the group at each time point. About 70% of the rats that were pretreated orally, did not develop any clinical symptoms and the other rats in this group were partially protected. The weight of the animals corroborated with the clinical evaluation. Control, nontreated rats, lost about 10 g per rat between 4 and 8 weeks after EAMG induction, whereas rats pretreated orally with the recombinant fragments gained about 10 g per rat during this time interval (Fig. 11). T-cell response to AChR as well as anti-rat AChR antibody titers were also reduced following oral treatment (Fig. 12).

In the second part of the experiment, the potential of oral administration of H α 1-205+p3A to modulate an ongoing disease (in rats immunized with AChR) was investigated. In this experiment, a denatured preparation of H α 1-205+p3A (designated denH α 1-215+p3A) was employed for oral treatment of sick rats. Denaturation of H α 1-205+p3A was performed in 6M guanidine HCL, followed by reduction with 0.1M β -mercaptoethanol and carboxymethylation with 0.15M iodoacetamide. Rats with a mild form of EAMG (clinical score of about 1) were pooled and divided randomly into two groups. Rats in the experimental group were fed 7 times with three days interval, each time with 0.3 mg of denH α 1-205+p3A per rat, and rats in the control group were fed with ovalbumin. The rats were evaluated weekly for clinical symptoms and for their body weight. As seen in Fig. 13, the disease was arrested in the rats treated orally with the recombinant fragment and their body weight increased. On the other hand the disease progressed in rats of the control group and the rats lost weight gradually.

These protection and suppression effects on EAMG shown in the above Examples, indicate that the polypeptides of the invention affect the autoimmune response to AChR in a manner that may be employed for immunotherapy of myasthenia gravis. Thus, the nasal or oral route of administration could provide a convenient therapeutic modality in humans.

5

REFERENCES

1. Aharonov, A., Abramsky, O., Tarrab-Hazdai, R. and Fuchs, S. (1975). Humoral antibodies to acetylcholine receptor in patients with myasthenia gravis. *Lancet* 2: 340.
2. Asher, O., Neumann, D. and Fuchs, S. (1988). Increased levels of acetylcholine
10 receptor α -subunit mRNA in experimental autoimmune myasthenia gravis. *FEBS Lett* 233: 277-81.
3. Asher, O., Kues, W. A., Witzemann, V., Tzartos, S. J., Fuchs, S. and Souroujon, M. C. (1993). Increased gene expression of acetylcholine receptor and myogenic factors in passively transferred experimental autoimmune myasthenia gravis. *J Immunol* 151: 6442-50.
- 15 4. Ausubel et al., eds., (1993) "Current Protocols in Molecular Biology" in *Current Protocols*.
5. Balass, M., Heldman, Y., Cabilly, S., Givol, D., Katchalski, K. E. and Fuchs, S. (1993). Identification of a hexapeptide that mimics a conformation-dependent binding site of
20 acetylcholine receptor by use of a phage-epitope library. *Proc Natl Acad Sci U S A* 90: 10638-42.
6. Barchan, D., Kachalsky, S., Neumann, D., Vogel, Z., Ovadia, M., Kochba, E. and Fuchs, S. (1992). How does the mongoose fight the snake: the binding site of the mongoose acetylcholine receptor. *Proc. Nat. Acad. Sci. USA* 89: 7717-7721.
7. Bartfeld, D. and Fuchs, S. (1978). Specific immunosuppression of experimental
25 autoimmune myasthenia gravis by denatured acetylcholine receptor. *Proc. Natl. Acad. Sci. USA* 75: 4006-4010.
8. Beeson, D., Morris, A., Vincent, A. and Newson-Davis, J. (1990). The human muscle nicotinic acetylcholine receptor α -subunit exists as two isoforms: a novel exon. *EMBO J.* 9: 2101-2106.
9. Changeux, J. P., Devillers-Thiery, A. and Chemouilli, P. (1984). The Acetylcholine
30 receptor: an allosteric protein. *Science*. 225: 1335-1345.
10. Dick, A. D., Cheng, Y. F., McKinnon, A., Liversidge, J. and Forrester, J. V. (1993). Nasal administration of retinal antigens suppresses the inflammatory response in experimental allergic uveoretinitis. A preliminary report of intranasal induction of tolerance with retinal
35 antigens. *Br J Ophthalmol* 77: 171-5.
11. Drachman, D. B. (1994). Myasthenia gravis. *N Engl J Med* 330: 1797-810.

12. Karlin, A. (1980) .Molecular properties of the acetylcholine receptors. in *In The Cell Surface and Neuronal Function* . G. Poste, C.W.Cotman and G.L. Nicolson (eds), 191-260.

13. Lennon, V.A., Lambert, E.H., Leiby, K.R., Okarma, T.B. and Talib, S. (1991).
Recombinant human acetylcholine receptor α -subunit induces chronic experimental
5 autoimmune myasthenia gravis. *J. Immunol.* 146, 2245-2248.

14. Lindstrom, J.M., Einarson, B.L., Lennon, V.A. and Seybold, M.E. (1976).
Pathological mechanisms in experimental autoimmune myasthenia gravis. I. Immunogenicity
of syngeneic muscle acetylcholine receptor and quantitative extraction of receptor and
antibody-receptor complexes from muscles of rats with experimental autoimmune myasthenia
10 gravis. *J. Exp. Med.* 144, 726-738.

15. Lindstrom, J.M., Engel, A.G., Seybold, M.E., Lennon, V.A. and Lambert, E.H.
(1976a). Pathological mechanisms in experimental autoimmune myasthenia gravis. II. Passive
transfer of experimental autoimmune myasthenia gravis in rats with anti-acetylcholine
receptor antibodies. *J. Exp. Med.* 144, 739-753.

16. Loutrari, H., Kokla, A. and Tzartos, S. J. (1992). Passive transfer of experimental
myasthenia gravis via antigenic modulation of acetylcholine receptor. *Eur J Immunol* 22:
2449-52.

17. Loutrari, H., Tzartos, S. J. and Claudio, T. (1992a). Use of Torpedo-mouse hybrid
acetylcholine receptors reveals immunodominance of the alpha subunit in myasthenia gravis
20 antisera. *Eur J Immunol* 22: 2949-56.

18. Ma, C. G., Zhang, G. X., Xiao, B. G., Link, J., Olsson, T. and Link, H. (1995).
Suppression of experimental autoimmune myasthenia gravis by nasal administration of
acetylcholine receptor. *J Neuroimmunol* 58: 51-60.

19. Mochly-Rosen, D. and Fuchs, S. (1981). Monoclonal anti-acetylcholine receptor
25 directed against the cholinergic binding site. *Biochemistry* 20: 5920-5924.

20. Neumann, D., Gershoni, J. M., Fridkin, M. and Fuchs, S. (1985). Antibodies to
synthetic peptides as probes for the binding site on the alpha subunit of the acetylcholine
receptor. *Proc Natl Acad Sci U S A* 82: 3490-3.

21. Newland, C. F., Beeson, D., Vincent, A. and Newsom-Davis, J. (1995). Functional
30 and non-functional isoforms of the human muscle acetylcholine receptor. *J. Physiol.* 489:
767-778.

22. Noda, M., Furutani, Y., Takahashi, H., Toyosato, M., Tanabe, T., Shimizu, S.,
Kikuyotani, S., Kayano, T., Hirose, T., Inayama, S. and Numa, S. (1983). Cloning and
sequence analysis of calf cDNA and human genomic DNA encoding α -subunit precursor of
35 muscle acetylcholine receptor. *Nature* 305, 818-823.

23. Patrick, J. and Lindstrom, J. M. (1973). Autoimmune response to acetylcholine
receptor. *Science* 180: 871-872.

24. Sambrook et al., eds. (1989) "Molecular Cloning: A Laboratory Manual", 2nd ed., Cold Spring Harbor Press.

25. Schoepfer, R., Luther, M. and Lindstrom, J. (1988). The human medulloblastoma cell line TE671 expresses a muscle-like acetylcholine receptor. Cloning of the alpha-subunit cDNA. *FEBS Lett* 226: 235-40.

26. Smith, D. B. and Johnson, K. S. (1988). Single-step purification of polypeptides expressed in *Escherichia coli* as fusions with glutathione S-transferase. *Gene* 67: 31-40.

27. Sophianos, D. and Tzartos, S.J. (1989). Fab fragments of monoclonal antibodies protect the human acetylcholine receptor against antigenic modulation caused by myasthenic sera. *J. Autoimmunity* 2, 777-789.

28. Souroujon, M.C., Pizzighella, S. Mochly-Rosen, D. and Fuchs, S. (1985). Antigenic specificity of acetylcholine receptor in developing muscle: Studies with monoclonal antibodies. *J. of Neuroimmunology*, 8; 159-166.

29. Souroujon, M. C., Pachner, A. R. and Fuchs, S. (1986). The treatment of passively transferred experimental myasthenia with anti- idiotypic antibodies. *Neurology* 36: 622-5.

30. Souroujon, M. C., Carmon, S. and Fuchs, S. (1992). Modulation of anti-acetylcholine receptor antibody specificities and of experimental autoimmune myasthenia gravis by synthetic peptides. *Immunol Lett* 34: 19-25.

31. Souroujon, M. C., Carmon, S. and Fuchs, S. (1993). Regulation of experimental autoimmune myasthenia gravis by synthetic peptides of the acetylcholine receptor. *Ann N Y Acad Sci* 681: 332-334.

32. Tzartos, S. and Lindstrom, J. (1980). Monoclonal antibodies used to probe acetylcholine receptor structure: localization of the main immunogenic region and detection of similarities between subunits. *Proc.Natl.Acad. Sci. USA* 77: 755.

33. Tzartos, S. J., Hochschwender, S., Vasquez, P. and Lindstrom, J. (1987). Passive transfer of experimental autoimmune myasthenia gravis by monoclonal antibodies to the main immunogenic region of the acetylcholine receptor. *J. Neuroimmunol.* 15: 185-194.

34. Weiner, H. L., Friedman, A., Miller, A., Khoury, S. J., al, S. A., Santos, L., Sayegh, M., N, u. R., Trentham, D. E. and Hafler, D. A. (1994). Oral tolerance: immunologic mechanisms and treatment of animal and human organ-specific autoimmune diseases by oral administration of autoantigens. *Annu Rev Immunol* 12: 809-837.

35. Wilson, P. T., Lentz, T. L. and Hawrot, E. (1985). Determination of the primary amino acid sequence specifying the α -bungarotoxin binding site on the α -subunit of the acetylcholine receptor from *Torpedo californica*. *Proc. Natl. Acad. Sci. USA.* 82: 8790-8794.

CLAIMS

1. A polypeptide capable of modulating the autoimmune response of an individual to acetylcholine receptor, said polypeptide being selected from the group consisting of:

(i) a polypeptide corresponding to amino acid residues 1-210 of the human acetylcholine receptor (hAChR) α -subunit sequence depicted in Fig.1 (H α 1-210), in which is inserted, between amino acid residues 58 and 59, a sequence of 25 amino acid residues encoded by the p3A exon of the hAChR α -subunit gene, depicted in Fig. 2 (H α 1-210+p3A);

(ii) a polypeptide corresponding to amino acid residues 1-205 of the hAChR α -subunit sequence depicted in Fig.1 (H α 1-210), in which is inserted, between amino acid residues 58 and 59, a sequence of 25 amino acid residues encoded by the p3A exon of the hAChR α -subunit gene, depicted in Fig. 2 (H α 1-205+p3A);

(iii) a polypeptide corresponding to amino acid residues 1-121 of the hAChR α -subunit sequence depicted in Fig.1 (H α 1-121);

(iv) a polypeptide corresponding to amino acid residues 1-121 of the hAChR α -subunit sequence depicted in Fig.1, in which is inserted, between amino acid residues 58 and 59, a sequence of 25 amino acid residues encoded by the p3A exon of the hAChR α -subunit gene, depicted in Fig. 2 (H α 1-121+p3A);

(v) a polypeptide corresponding to amino acid residues 122-210 of the hAChR α -subunit sequence depicted in Fig.1 (H α 122-210);

(vi) a polypeptide as in (i) to (v) or the polypeptide H α 1-210 in which one or more amino acid residues have been added, deleted or substituted by other amino acid residues in a manner that the resulting polypeptide is capable of modulating the autoimmune response to acetylcholine receptor;

(vii) a fragment of a polypeptide as in (i) to (vi), which fragment is capable of modulating the autoimmune response to acetylcholine receptor;

(viii) a polypeptide comprising two or more fragments as in (vii) fused together with or without a spacer;

(ix) a polypeptide or a fragment as defined in (i)-(viii) or the polypeptide H α 1-210 fused to an additional polypeptide at its N- and/or C-terminal; and

(x) soluble forms, denatured forms, chemical derivatives and salts of a polypeptide or a fragment as defined in (i)-(ix).

2. A polypeptide according to claim 1 corresponding to amino acid residues 1-210 of the hAChR α -subunit sequence depicted in Fig.1, in which is inserted, between amino acid residues 58 and 59, a sequence of 25 amino acid residues encoded by the p3A exon of the hAChR α -subunit gene depicted in Fig. 2.

5

3. A polypeptide according to claim 1 corresponding to amino acid residues 1-205 of the hAChR α -subunit sequence depicted in Fig.1, in which is inserted, between amino acid residues 58 and 59, a sequence of 25 amino acid residues encoded by the p3A exon of the hAChR α -subunit gene depicted in Fig. 2.

10

4. A polypeptide according to claim 1, corresponding to amino acid residues 1-121 of the hAChR α -subunit sequence depicted in Fig.1.

5. A polypeptide according to claim 1, corresponding to amino acid residues 1-121 of the hAChR α -subunit sequence depicted in Fig.1, in which is inserted, between amino acid residues 58 and 59, a sequence of 25 amino acid residues encoded by the p3A exon of the hAChR α -subunit gene depicted in Fig.2.

15

6. A polypeptide according to claim 1, corresponding to amino acid residues 122-210 of the hAChR α -subunit sequence depicted in Fig.1.

20

7. A polypeptide or a fragment according to claim 1(ix) wherein the additional polypeptide is glutathione S-transferase (GST) fused at the N-terminal of a polypeptide according to claim 1 (i) - (viii).

25

8. A DNA molecule coding for a polypeptide or a fragment according to claim 1.

9. A DNA molecule according to claim 8, being selected from the group consisting of:

(i) a DNA molecule comprising the sequence of nucleotides 1 to 630, depicted in Fig.1, in which the sequence of the p3A exon of the hAChR α -subunit gene, depicted in Fig. 2, is inserted between nucleotides 174 and 175;

30

(ii) a DNA molecule comprising the sequence of nucleotides 1 to 615, depicted in Fig.1, in which the sequence of the p3A exon of the hAChR α -subunit gene, depicted in Fig. 2, is inserted between nucleotides 174 and 175;

(iii) a DNA molecule comprising the sequence of nucleotides 1 to 363 depicted in Fig.1;

(iv) a DNA molecule comprising the sequence of nucleotides 1 to 363 depicted in Fig.1, in which the sequence of the p3A exon of the hAChR α -subunit gene, depicted in Fig. 2, is inserted between nucleotides 174 and 175;

(v) a DNA molecule comprising the sequence of nucleotides 364 to 630 depicted in Fig.1;

(vi) DNA molecules which are degenerate, as a result of the genetic code, to the DNA sequences of (i) to (v) and which code for a polypeptide coded for by any one of the DNA sequences of (i) to (v);

(vii) a DNA molecule having a coding nucleotide sequence which is at least 70% homologous to any one of the DNA sequences of (i) to (vi) or to the DNA sequence coding for H α 1-210;

(viii) a DNA molecule as in (i) to (v) or the DNA molecule coding for H α 1-210 in which one or more codons has been added, replaced or deleted in a manner that the polypeptide coded for by said sequence is capable of modulating the autoimmune response to acetylcholine receptor;

(ix) a fragment of a DNA molecule as in (i)-(viii) which codes for a polypeptide capable of modulating the autoimmune response to acetylcholine receptor;

(x) a DNA molecule comprising two or more fragments of (ix) fused together with or without a spacer, and which codes for a polypeptide capable of modulating the autoimmune response to acetylcholine receptor; and

(xi) a DNA molecule comprising a nucleic acid sequence as defined in (i)-(x) or the DNA sequence coding for H α 1-210 fused to additional coding DNA sequences at its 3' and/or 5' end.

10. A DNA molecule according to claim 9 which comprises the sequence of nucleotides 1 to 630, depicted in Fig.1, in which the sequence of the p3A exon of the hAChR α -subunit gene, depicted in Fig.2, is inserted between nucleotides 174 and 175, said DNA molecule coding for the polypeptide according to claim 2.

11. A DNA molecule according to claim 9 which comprises the sequence of nucleotides 1 to 615, depicted in Fig.1, in which the sequence of the p3A exon of the hAChR α -subunit gene, depicted in Fig.2, is inserted between nucleotides 174 and 175, said DNA molecule coding for the polypeptide according to claim 2.

5

12. A DNA molecule according to claim 9 which comprises the sequence of nucleotides 1 to 363 of the DNA sequence depicted in Fig.1, said DNA molecule coding for the polypeptide according to claim 3.

10

13. A DNA molecule according to claim 9 which comprises the sequence of nucleotides 1 to 363 of the DNA sequence depicted in Fig.1, in which a nucleic acid sequence of the p3A exon of the hAChR α -subunit gene, depicted in Fig. 2, is inserted between nucleotides 174 and 175, said DNA molecule coding for the polypeptide according to claim 4.

15

14. A DNA molecule according to claim 9 which comprises the sequence of nucleotides 364 to 630 of the DNA sequence depicted in Fig.1, said DNA molecule coding for the polypeptide according to claim 5.

15. A DNA molecule according to claim 9 (xi) wherein said additional nucleic acid sequence codes for glutathione S-transferase (GST) and is fused at the 5' end, said fused DNA molecule coding for a polypeptide according to claim 7.

20

16. A replicable expression vehicle comprising a DNA molecule according to any one of claims 8-15.

25

17. A prokaryotic or eukaryotic host cell transformed by a replicable expression vehicle of claim 16.

18. A process for preparing a polypeptide of any one of claims 1-7, comprising:

30

- (i) culturing a host cell of claim 17 under conditions promoting expression, and
- (ii) isolating the expressed polypeptide.

19. A process according to claim 17, wherein the expressed polypeptide is a fused polypeptide.

20. A pharmaceutical composition comprising a pharmaceutically acceptable carrier
5 and a polypeptide selected from a polypeptide according to any one of claims 1 to 7 and a polypeptide comprising the amino acid residues 1-210 of the hAChR α -subunit depicted in Fig.1, soluble forms, denatured forms, salts and chemical derivatives thereof.

21. A pharmaceutical composition according to claim 20 for alleviation and/or
10 treatment of myasthenia gravis.

22. A pharmaceutical composition according to claim 21 for nasal or oral administration.

23. A method for alleviation and/or treatment of myasthenia gravis which comprises
15 administering to an individual in need thereof, an effective amount of a polypeptide according to any one of claims 1 to 7 or a polypeptide comprising the amino acid residues 1-210 of the hAChR α -subunit depicted in Fig.1, soluble forms, denatured forms, salts and chemical derivatives thereof.

24. A pharmaceutical composition according to claim 20 for diagnosis of myasthenia
20 gravis.

25. A method for diagnosis of myasthenia gravis comprising:

25 (i) incubating one or more polypeptides selected from a polypeptide according to any one of claims 1 to 7 and a polypeptide comprising the amino acid residues 1-210 of the hAChR α -subunit depicted in Fig.1, soluble forms, denatured forms, salts and chemical derivatives thereof, with a serum aliquote from an individual; and

(ii) determining the amount of the anti-AChR antibodies in the serum bound to
30 said polypeptide of (i);

whereby detection of anti-AChR titers indicates the presence of myasthenia gravis.

1/12

1	TCC	GAA	CAT	GAG	ACC	CGT	CTG	GTG	GCA	AAG	CTA	TTT	AAA	GAC	TAC	45
1	Ser	Glu	His	Glu	Thr	Arg	Leu	Val	Ala	Lys	Leu	Phe	Lys	Asp	Tyr	15
46	AGC	AGC	GTG	GTG	CGG	CCA	GTG	GAA	GAC	CAC	CGC	CAG	GTC	GTG	GAG	90
16	Ser	Ser	Val	Val	Arg	Pro	Val	Glu	Asp	His	Arg	Gln	Val	Val	Glu	30
91	GTC	ACC	GTG	GGC	CTG	CAG	CTG	ATA	CAG	CTC	ATC	AAT	GTG	GAT	GAA	135
31	Val	Thr	Val	Gly	Leu	Gln	Leu	Ile	Gln	Leu	Ile	Asn	Val	Asp	Glu	45
136	GTA	AAT	CAG	ATC	GTG	ACA	ACC	AAT	GTG	CGT	CTG	AAA	CAG	CAA	TGG	180
46	Val	Asn	Gln	Ile	Val	Thr	Thr	Asn	Val	Arg	Leu	Lys	Gln	Gln	Trp	60
181	GTG	GAT	TAC	AAC	CTA	AAA	TGG	AAT	CCA	GAT	GAC	TAT	GGC	GGT	GTG	225
61	Val	Asp	Tyr	Asn	Leu	Lys	Trp	Asn	Pro	Asp	Asp	Tyr	Gly	Gly	Val	75
226	AAA	AAA	ATT	CAC	ATT	CCT	TCA	GAA	AAG	ATC	TGG	CGC	CCA	GAC	CTT	270
76	Lys	Lys	Ile	His	Ile	Pro	Ser	Glu	Lys	Ile	Trp	Arg	Pro	Asp	Leu	90
271	GTT	CTC	TAT	AAC	GAT	GCA	GAT	GGT	GAC	TTT	GCT	ATT	GTC	AAG	TTC	315
91	Val	Leu	Tyr	Asn	Asp	Ala	Asp	Gly	Asp	Phe	Ala	Ile	Val	Lys	Phe	105
316	ACC	AAA	GTG	CTC	CTG	CAG	TAC	ACT	GGC	CAC	ATC	ACG	TGG	ACA	CCT	360
106	Thr	Lys	Val	Leu	Leu	Gln	Tyr	Thr	Gly	His	Ile	Thr	Trp	Thr	Pro	120
361	CCA	GCC	ATC	TTT	AAA	AGC	TAC	TGT	GAG	ATC	ATC	GTC	ACC	CAC	TTT	405
121	Pro	Ala	Ile	Phe	Lys	Ser	Tyr	Cys	Glu	Ile	Ile	Val	Thr	His	Phe	135
406	CCC	TTT	GAT	GAA	CAG	AAC	TGC	AGC	ATG	AAG	CTG	GGC	ACC	TGG	ACC	450
136	Pro	Phe	Asp	Glu	Gln	Asn	Cys	Ser	Met	Lys	Leu	Gly	Thr	Trp	Thr	150
451	TAC	GAC	GGC	TCT	GTC	GTG	GCC	ATC	AAC	CCG	GAA	AGC	GAC	CAG	CCA	495
151	Tyr	Asp	Gly	Ser	Val	Val	Ala	Ile	Asn	Pro	Glu	Ser	Asp	Gln	Pro	165
496	GAC	CTG	AGC	AAC	TTC	ATG	GAG	AGC	GGG	GAG	TGG	GTG	ATC	AAG	GAG	540
166	Asp	Leu	Ser	Asn	Phe	Met	Glu	Ser	Gly	Glu	Trp	Val	Ile	Lys	Glu	180
541	TCC	CGG	GGC	TGG	AAG	CAC	TCC	GTG	ACC	TAT	TCC	TGC	TGC	CCC	GAC	585
181	Ser	Arg	Gly	Trp	Lys	His	Ser	Val	Thr	Tyr	Ser	Cys	Cys	Pro	Asp	195
586	ACC	CCC	TAC	CTG	GAC	ATC	ACC	TAC	CAC	TTC	GTC	ATG	CAG	CGC	CTG	630
196	Thr	Pro	Tyr	Leu	Asp	Ile	Thr	Tyr	His	Phe	Val	Met	Gln	Arg	Leu	210

Fig 1

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GGT GAC ATG GTA GAT CTG CCA CGC CCC AGC TGC GTG ACT TTG GGA
Gly Asp Met Val Asp Leu Pro Arg Pro Ser Cys Val Thr Leu Gly

GTT CCT TTG TTT TCT CAT CTG CAG GAT GAG
Val Pro Leu Phe Ser His Leu Gln Asp Glu

Fig 2

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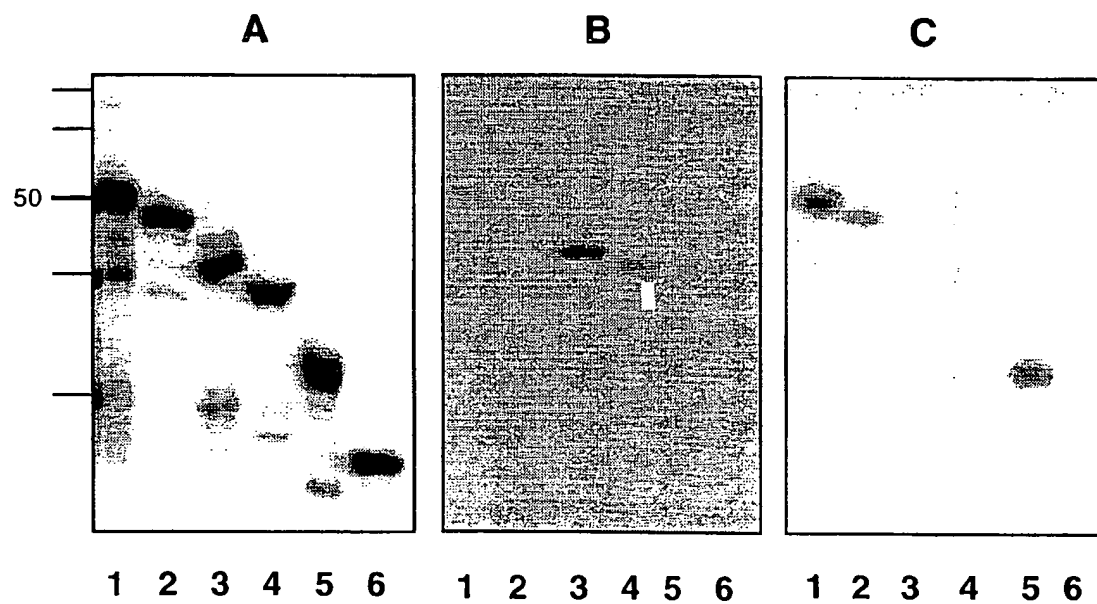


Fig 3.

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Fig 4

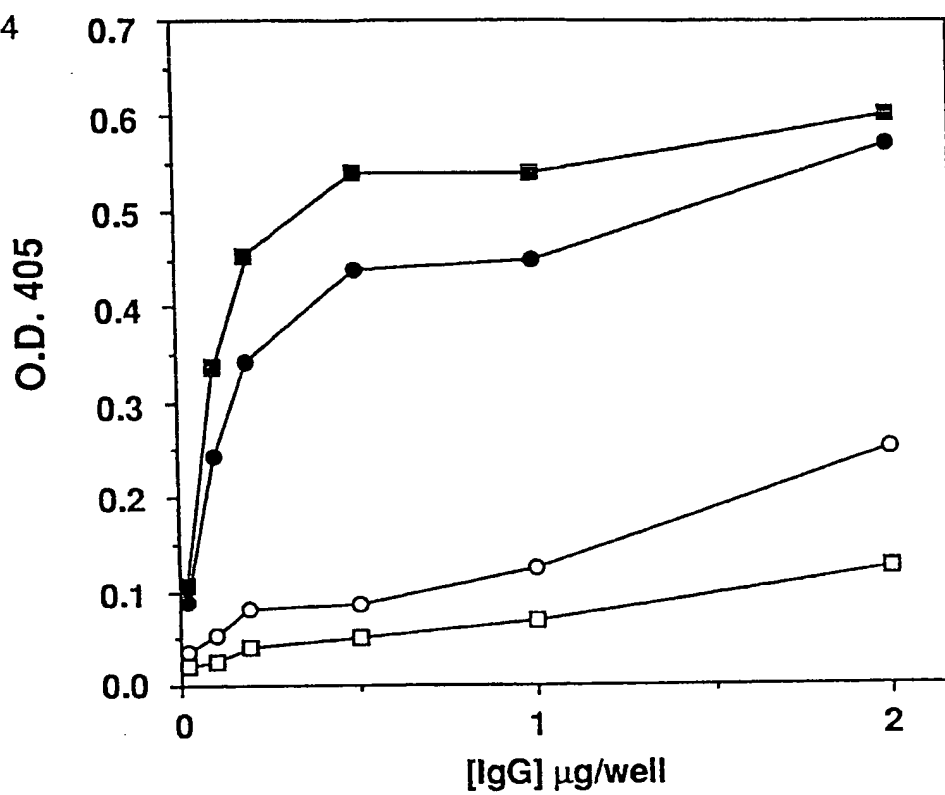
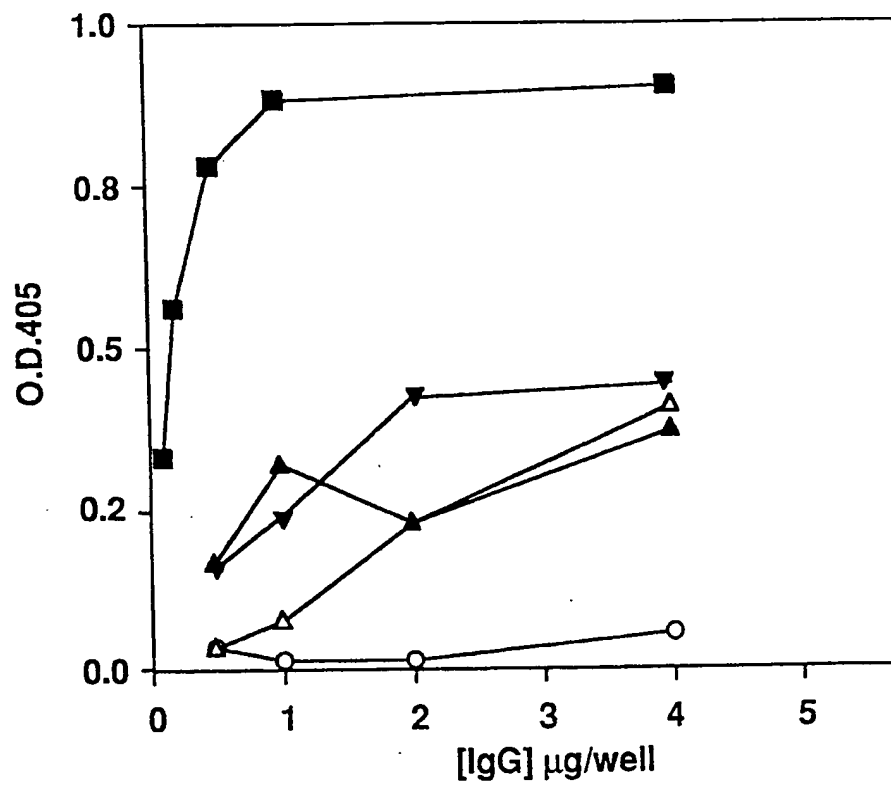


Fig 5



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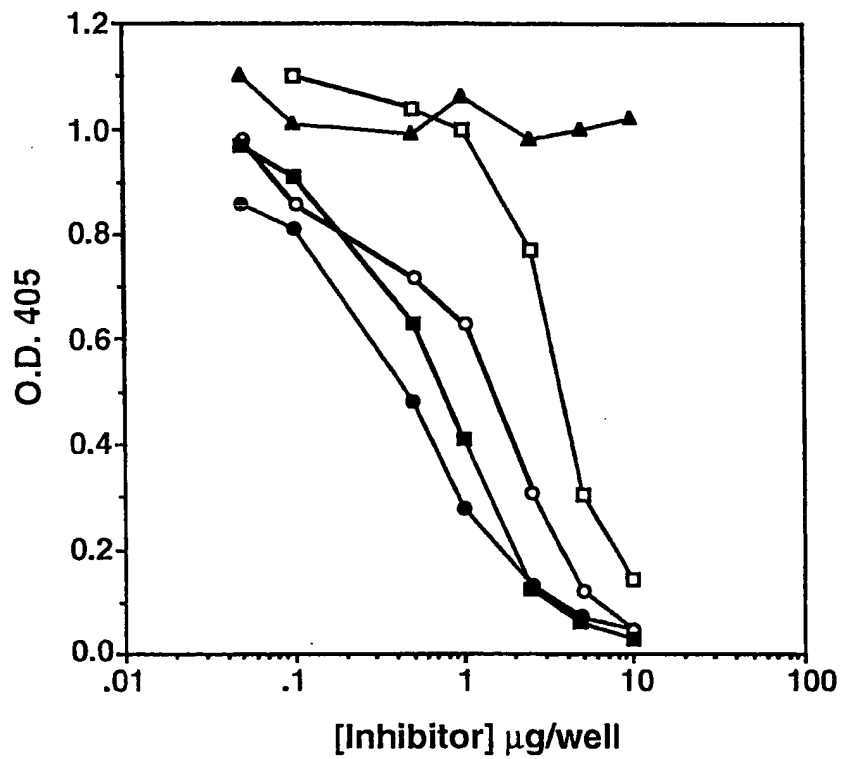


Fig 6

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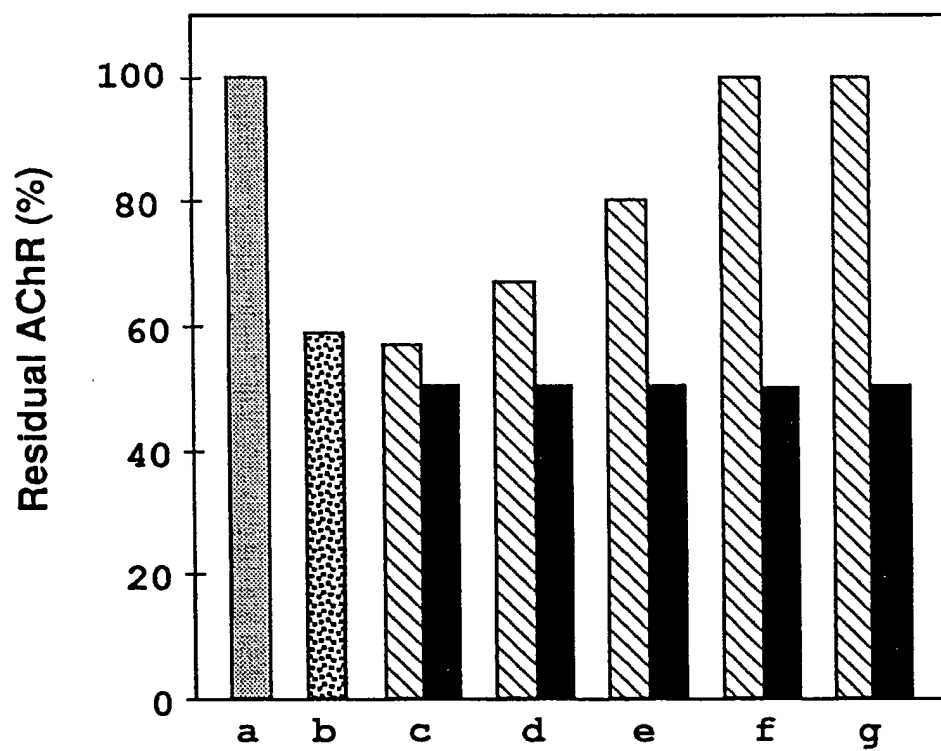


Fig7

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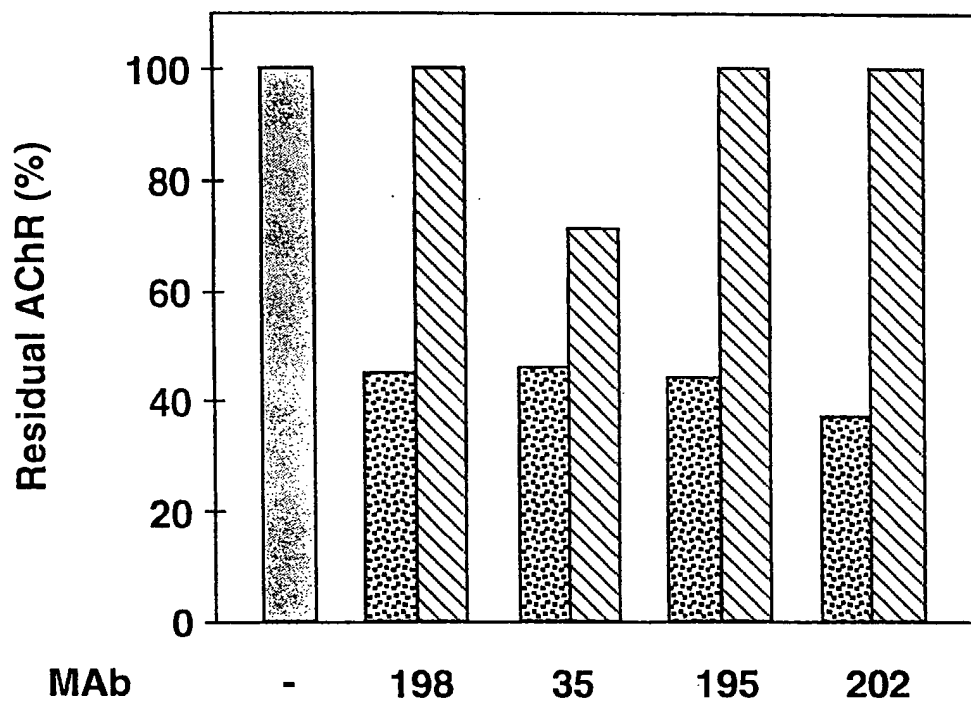


Fig 8

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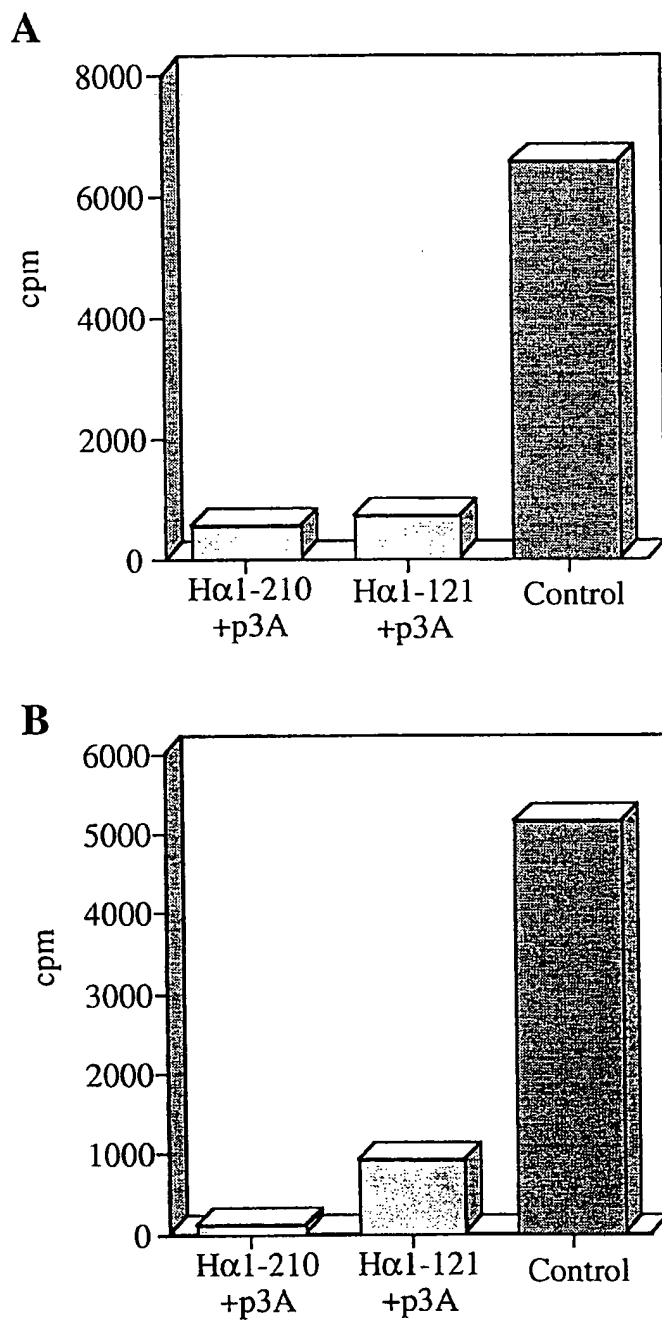


Fig.9

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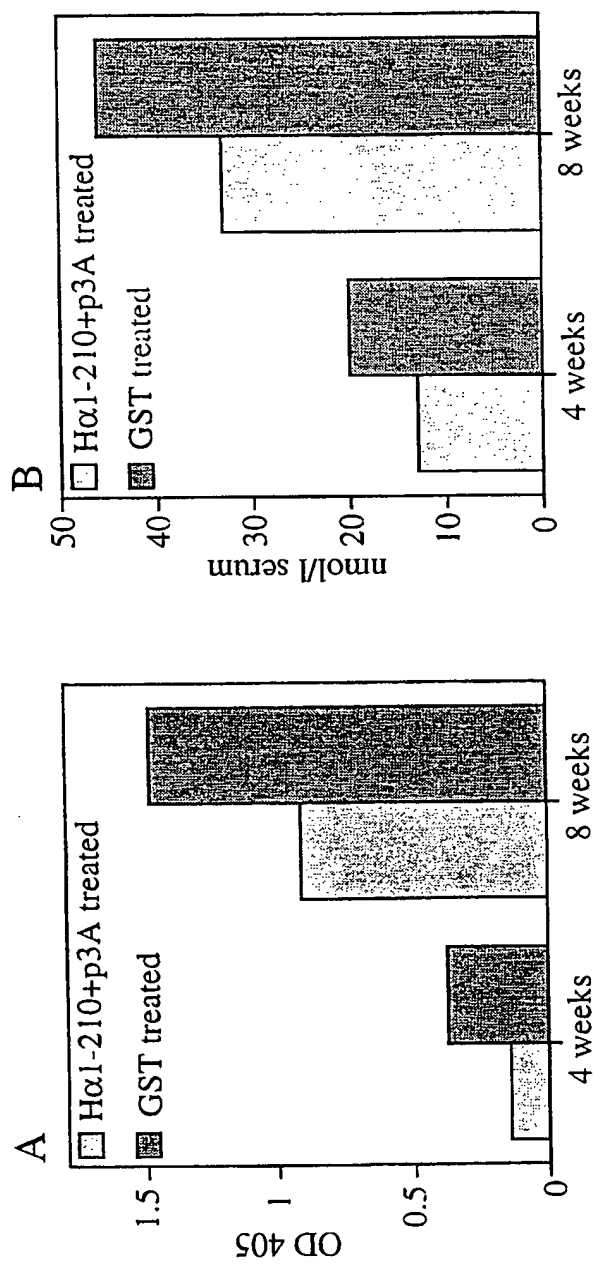
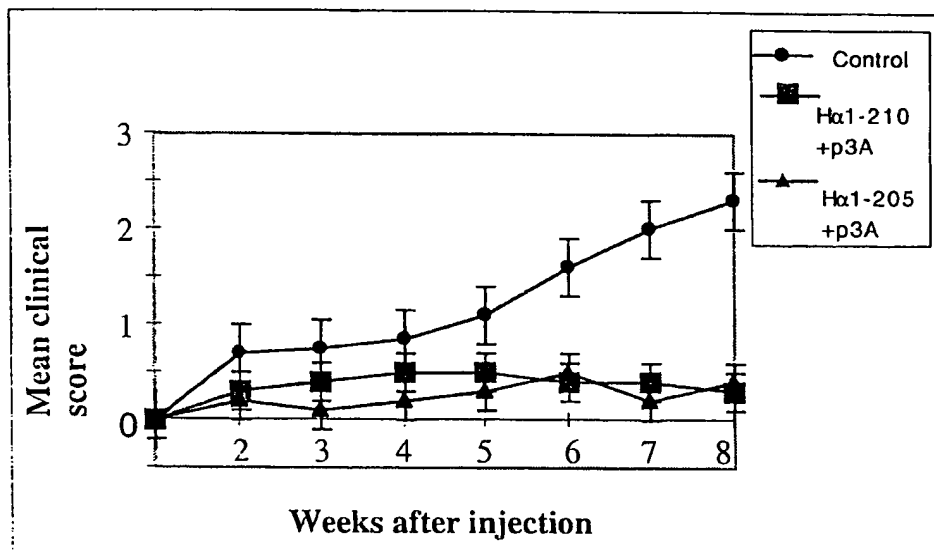


Fig. 10

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A



B

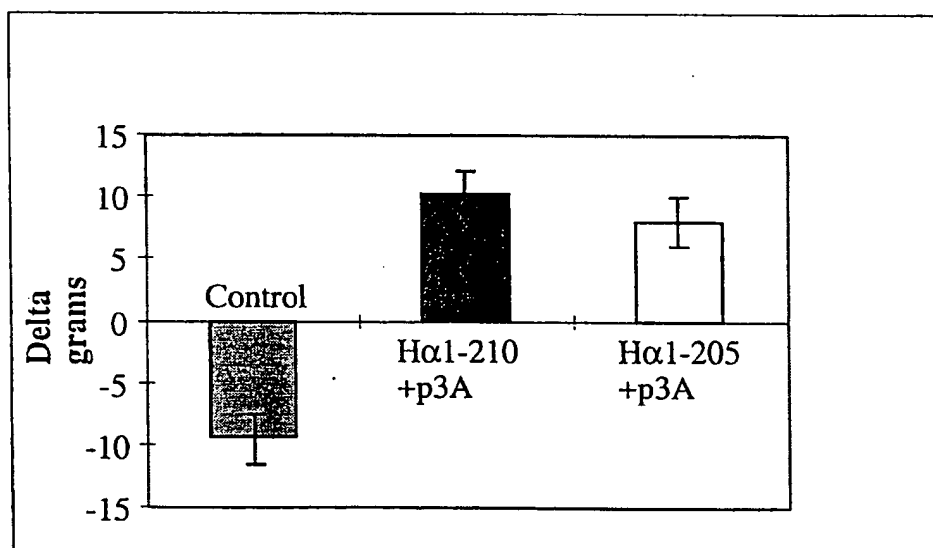
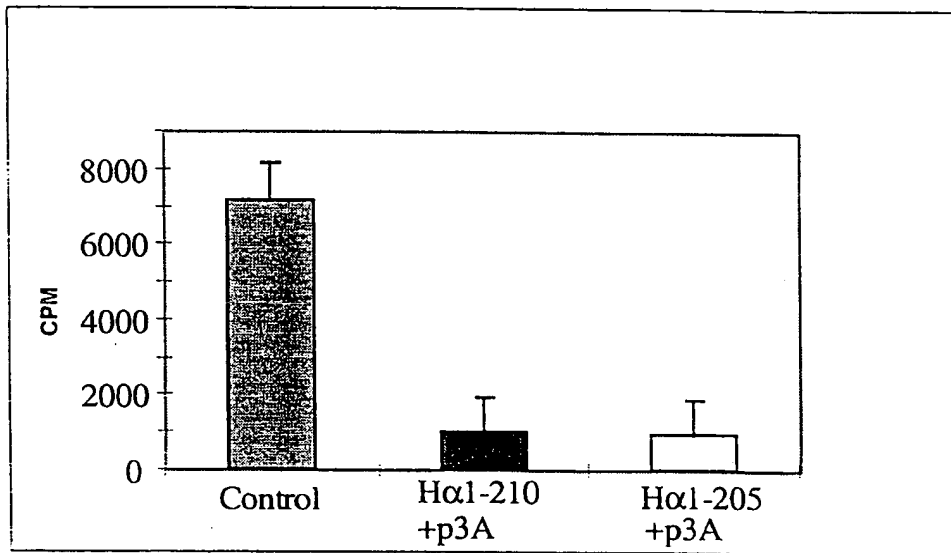
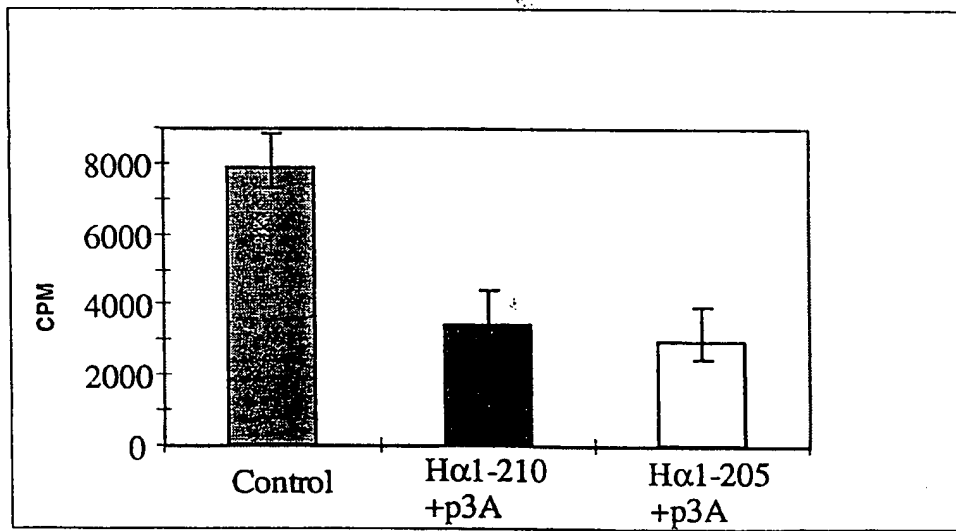


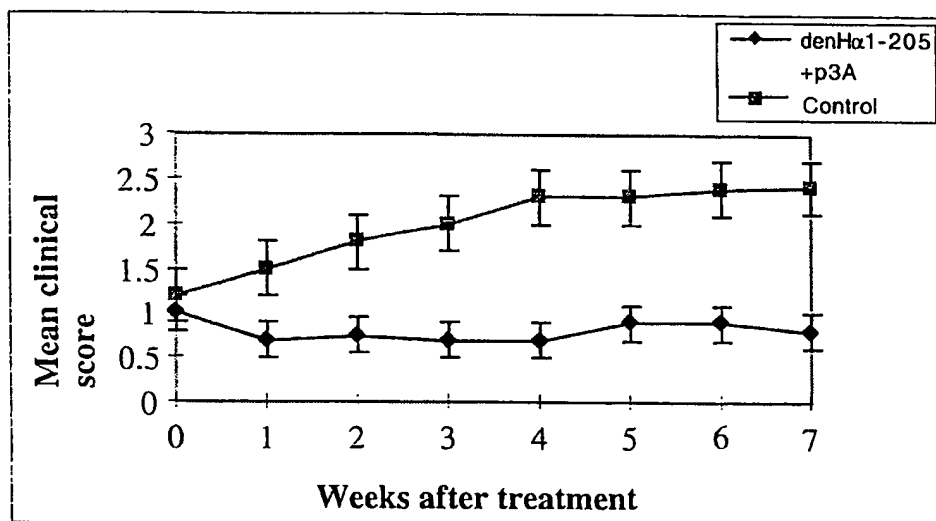
Fig.11

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A**B****Fig.12**

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A



B

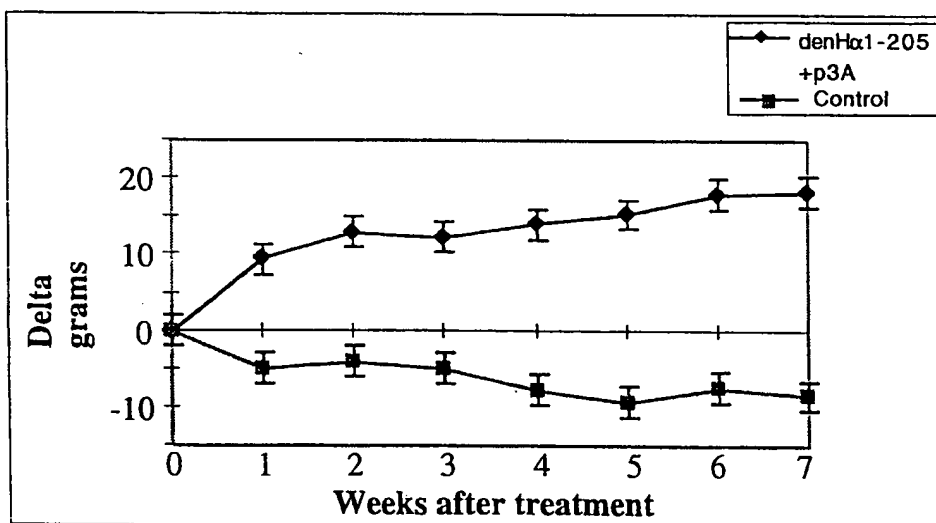


Fig. 13

INTERNATIONAL SEARCH REPORT

Int'l Application No

PCT/IL 98/00211

A. CLASSIFICATION OF SUBJECT MATTER

IPC 6 C12N15/12 C07K14/705 C12N15/70 C12N1/21 A61K38/17
G01N33/53

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 C12N C07K A61K G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	<p>US 5 578 496 A (M. ZOUHAIR ATASSI ET AL.) 26 November 1996 see column 5, line 20 - column 6, line 63 see column 9, line 4 - column 10, line 65 see column 14, line 19 - line 31; examples II-V</p> <p style="text-align: center;">--- -/--</p>	<p>1, 7-9, 15-25</p>

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier document but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
"&" document member of the same patent family

Date of the actual completion of the international search

17 September 1998

Date of mailing of the international search report

01/10/1998

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Authorized officer

Montero Lopez, B

INTERNATIONAL SEARCH REPORT

International Application No

PCT/IL 98/00211

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	<p>DAVID BEESON ET AL.: "The human muscle nicotinic acetylcholine receptor alpha-subunit exists as two isoforms: a novel exon"</p> <p>EMBO JOURNAL, vol. 9, no. 7, July 1990, pages 2101-2106, XP002077782</p> <p>EYNHAM, OXFORD GB cited in the application see abstract see page 2101, right-hand column, paragraph 4 - page 2102, right-hand column, paragraph 1</p> <p>---</p>	1-25
A	<p>TALIB S ET AL: "Cloning and expression in Escherichia coli of a synthetic gene encoding the extracellular domain of the human muscle acetylcholine receptor alpha-subunit."</p> <p>GENE, (1991 FEB 15) 98 (2) 289-93. JOURNAL CODE: FOP. ISSN: 0378-1119., XP002077783 Netherlands see the summary see page 289, right-hand column, paragraph 2 - page 293, left-hand column, paragraph 1</p> <p>---</p>	1-25
A	<p>SANO M ET AL: "Identification of three extended antibody-binding segments in recombinant human muscle acetylcholine receptor alpha subunit extracellular domain 1-210."</p> <p>INTERNATIONAL IMMUNOLOGY, (1991 OCT) 3 (10) 983-9. JOURNAL CODE: AY5. ISSN: 0953-8178., XP002077784 ENGLAND: United Kingdom see abstract see page 984, right-hand column, paragraph 2 - page 985, left-hand column, paragraph 1 see page 986, left-hand column, paragraph 3 - page 987, left-hand column, paragraph 1</p> <p>---</p>	1-25
A	<p>DORA BARCHAN ET AL.: "The binding site of the Nicotinic Acetylcholine receptor in animal species resistant to alpha-Bungarotoxin"</p> <p>BIOCHEMISTRY, vol. 34, no. 28, 18 July 1995, pages 9172-9176, XP002077785 EASTON, PA US see page 9173, right-hand column, paragraph 3 - page 9176, left-hand column, paragraph 2</p> <p>---</p>	1-25
	<p>---</p> <p>-/--</p>	

INTERNATIONAL SEARCH REPORT

International Application No

PCT/IL 98/00211

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P, X	<p>BARCHAN D ET AL: "Modulation of the anti-acetylcholine receptor response and experimental autoimmune myasthenia gravis by recombinant fragments of the acetylcholine receptor." EUROPEAN JOURNAL OF IMMUNOLOGY, (1998 FEB) 28 (2) 616-24. JOURNAL CODE: EN5. ISSN: 0014-2980., XP002077786 GERMANY: Germany, Federal Republic of see abstract see page 617, left-hand column, paragraph 2 - right-hand column, paragraph 1 see page 618, right-hand column, paragraph 3 - page 621, right-hand column, paragraph 2</p> <p>-----</p>	1,2, 4-10, 12-25

INTERNATIONAL SEARCH REPORT

International application No.

PCT/IL 98/00211

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
Remark: Although claim 23 is directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compounds.
2. ☐ Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/IL 98/00211

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US 5578496 A	26-11-1996	AU 3423293 A WO 9312145 A	19-07-1993 24-06-1993